Pharmaceutical Development Processes

James R H Scarr, MEng

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Department of Biochemical Engineering
University College London
Torrington Place
London
WC1E 7JE

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

The process of developing pharmaceuticals requires expertise from numerous different scientific areas. Four separate studies have been undertaken on Pharmaceuticals Testing, Process Development, Business Strategy and Process Validation within this industry.

New pharmaceuticals generally require multi-step reactions, which increasingly feature the involvement of biological synthesis to improve the optical purity and thus efficacy and safety of the drug. Two of the problems with employing biological synthesis are the high level of inhibition observed and the potential difficulty with which these batch based reactions are combined with semi-continuous chemical synthesis. The first study characterises the inhibition of CHMO, a promising oxygenase enzyme, with the aid of flow cytometry using different systems:

- A range of substrates, based around the natural substrate cyclohexanone but with differing ring size and increasing chain length.
- Different CHMO catalysed reactions - isolated enzyme, free cell and immobilised whole cell.

As expected, reactions with CHMO expressed in *E. coli* TOP10 [pQR239] in their immobilised form reduced the observed reaction rate. Unexpectedly, for the more rapidly converted substrates (generally those closest to cyclohexanone), immobilisation was found to increase the inhibition observed. It has been postulated that this is due to an oxygen shortage for maintaining cell metabolism and a time based inhibitory effect.

Advantages of immobilised cells are that they can be rapidly removed from the reaction broth allowing greater integration with other processes and can be recycled for multiple re-use. To facilitate their industrial use, the large scale production of immobilised whole cells is required. Whilst immobilised cell reactions are industrially employed, how such large quantities of immobilised cells are produced is yet to be reported. The feasibility of immobilisation of oxygenase expressing cells has been assessed in this first study, using the flow cytometry as a tool for assessing cell damage in the key step of cell separation.

Within the pharmaceutical development process drug molecules are rigorously tested in clinical trials. However the metabolites likely to be produced in the body, which may be active (and preferable drug candidates to the parent molecule) or toxic (and thus responsible for the failure of the drug in the final stages of clinical trials) are often ignored. Within the human body the oxygenase enzymes Cytochrome P450s (CYPs) are responsible for the primary metabolism of more than 90% of drugs. The second study assesses different methods of identifying the CYP responsible for metabolism and discusses the importance of being able to produce gram scale quantities of metabolites. This study indicated that the best currently feasible option of CYP identification is the employment of Bactosomes™ (individual CYP enzymes expressed in bacteria) with a selective inhibitor pre-screen.

The scientific complexity of the pharmaceutical development process makes effective strategic planning and decision making difficult. Whilst the necessity of business plans to enable companies to secure finance has helped scientists to gain an understanding of their market and associated business risks, business decisions such as when to invest and how much, often rely solely on the company's tolerance of risk, collective intuition and experience. The third study investigates the business strategy of the pharmaceutical development process. StrategyDynamics® modelling has been employed to create a living model of a start-up contract research organisation. The model demonstrates the advantages of being able to predict key resource bottlenecks, contrast different business decisions such as growth strategy and plan for future events and changes in technology and markets. This modelling can potentially save companies from expensive trial and error approaches and help to manage risk.

Regulatory pressure within the pharmaceutical development industry and the importance of validation is increasing. In the fourth study the application of Process Validation to the areas of pharmaceutical development process in the first three studies are investigated. For CHMO biocatalysis the reproducibility of immobilised experiments was assessed, for drug metabolite production the importance of change validation, i.e. assay robustness, was determined and for the Strategy Dynamics modelling an approach to validating the model has been detailed.

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1 Introduction

1.1 Enzymes

Enzymes are industrially used for a broad range of applications, from final products in detergents, animal feeds and analytical tests, to their use as processing aids in the paper and textile industries and their application in the brewing and dairy industries (Tramper, 1996). However, it is a further application of enzymes that is of interest in this work, namely their use in commercial biocatalysis in the pharmaceutical industry. Biocatalysis is the use of an enzyme, either in an isolated and potentially purified and immobilised form, a whole cell, or an immobilised whole cell to catalyse a specific chemical reaction in which defined substrate(s) are converted to defined product(s) (Woodley and Lilly, 1994).

1.1.1 Types of enzymes

Enzymes are subdivided by the Enzyme Commission into six different classes according to the type of reactions that they catalyse as shown in Table 1.1. The current version of the ENZYME database (http://www.expasy.ch/enzyme/) has 3912 active enzymes registered.

This project focuses on two oxygenase enzymes (i.e. oxidoreductases), cyclohexanone monooxygenase (CHMO) and cytochrome P450 3A4. Oxygenase enzymes and their role in biocatalysis are discussed in section 1.1.5.

Enzyme class (No. identified/ No. commercially available)	**Example of use**	**Reference**
EC1:Oxidoreductases *(650/90)* Interconverts ketones with alcohols, double bonds with single bonds etc.	Merck & Co have derived an anti-asthma drug Montelukast, using a selective ketone reductase.	Shafiee et al. (1998)
EC2:Transferases *(720/90)* Transfer acyl, phosphoryl, sugar, amino acid groups etc. e.g. Kinases, Transaminases.	Kinase inhibitors are potential drug candidates for the treatment of cancers.	Gallion and Qian (2005).
EC3: Hydrolases *(636/125)* Hydrolysis of esters, peptides, glycerides, anhydrides, etc.	Astra Zeneca has used a halo acid dehalogenase for the resolution of chloropropionic acid, used in the production of herbicides at a 2000 tonnes per year scale.	Taylor (1997)
EC4: Lyases *(255/35)* Addition to double bonds C=C (aspartase, fumarase), C=N, C=O (aldolases), etc.	The dietary supplement Malic acid is produced from fumaric acid, catalyzed by the enzyme Fumarase.	Presecki and Vasic-Racki (2005)
EC5: Isomerases *(120/6)* Various isomerizations, C=C bond migration, cis-trans racemizations, etc.	Various companies produce fructose from fructose-glucose isomerase. More than a million tonnes are produced per annum	Liese and Filho (1999)
EC6: Ligases *(80/5)* Formation of C-O, C-S, C-N and phosphoryl bonds	The cloning of DNA in molecular biology.	Sreedhara *et al.* (2004)

Table 1.1: The six enzyme classes and examples of their use. Adapted from Roberts *et al*, 1990.

1.1.2 The importance of enzymes

There are three main reasons why enzymes are of commercial interest. Firstly they can demonstrate high levels of product purity due to their ability to yield optically pure products, the importance of which is discussed in section 1.1.3. Secondly, they can perform reactions that chemical synthesis routes either cannot, or are unable to do with comparable optical purity. Thirdly they can significantly reduce the number of reactive steps required. They are also able to operate under mild conditions, thus being potentially more environmentally friendly and more suitable for the production of unstable compounds.

1.1.3 Chirality

A molecule is chiral when it has two different spatial arrangements, where one cannot be superimposed on its own mirror image. The two mirror image compounds are called enantiomers.

Chirality is a key factor in the efficacy of many drugs and agrochemicals, and the production of single enantiomers of chiral intermediates has therefore become increasingly important (Patel, 2001). The importance of chirality was recognized in 2001, when Prof. Barry Sharpless was awarded the Nobel Prize for his work on chirally catalyzed oxidation reactions.

The importance of chirality to the pharmaceutical industry is that different enantiomers of a chemical compound may have different biological activities. Chiral drugs can be classified depending upon the respective pharmacological activity of the two enantiomers:

1. Both enantiomers exhibit the same, or nearly the same, pharmacological activity (variations in potency may also exist).
2. Enantiomers have different pharmacological activity.
3. One enantiomer is responsible for all activity, the other being at best a waste product, or at worst potentially toxic or counter-active.

An example of the third class, which was arguably the driving force behind the push for enantiopure drugs, is Thalidomide. One enantiomer was found in the 1960s to create birth defects, whilst the other contained the desired pharmacological activity. It was only in 1998 that the United States Food and Drug Administration (FDA) approved the use of enantiopure Thalidomide to treat leprosy (Muller, 1997).

The Federal Drug Agency (FDA) and other agencies have now issued regulatory guidelines which state that in order to gain regulatory approval for a racemic drug, companies must provide pharmacological and toxicity data separately for each enantiomer (FDA policy statement for the development of new stereoisomeric drugs, 1992). Furthermore, previously licensed racemic drugs can be redeveloped as single enantiomer products to extend their patent protection (Stinson, 2000). These regulatory guidelines have provided pharmaceutical companies with a strong incentive to develop single enantiomer drugs due to the costs of providing data for two separate enantiomers. This has driven an expansion in the numbers of pharmaceuticals produced as single enantiomers over the last decade, a trend which looks set to continue. For example in 1999, single stereoisomer drugs represented 32 % of the worldwide pharmaceutical market, up 16 % from 1998, with sales of $115 billion (Stinson, 2000). This figure is expected to rise to 70% by the end of the 21st century (Schulze and Wubbolts, 1999)

Other prominent examples of the differences between enantiomers are (Liese, 2005):

- Limonene: (S)-Limonene smells like lemons, (R)-Limonene smells like oranges
- Propanolol: (S)-Propanolol is used for blood pressure treatment, (R)-Propanolol is a contraceptive.

There are a number of ways of achieving enantiopure products (Kelly *et al.*, 1998):

- Separating enantiomers by non-biocatalytic routes such are chromatography or crystallization (Atwell *et al.*, 2002) or by dynamic kinetic resolution in which one enantiomer of an achiral mixture is converted into the other by enzymatic racemisation (El Gihani and Williams, 1999; Schnell *et al.*, 2003; Gutierrez *et al.*, 2005)

- Biocatalytic resolution, whereby one enantiomer is virtually untouched whilst the other is converted into the desired enantiomerically pure product. Hydrolases are the most commonly used enzyme for industrial resolution (Schulze and Wubbolts, 1999).
- Applying asymmetric synthesis in which prochiral molecules are biotransformed by number of microorganisms or different catalysts to target molecules of high enantiomeric excess by selective introduction of chiral centres. An example of this method is the employment of oxygenases, discussed in section 1.1.5.

1.1.4 Advantages of enzymes versus chemistry

Some examples of different enzyme classes have already been noted in Table 1.1. Some advantages and disadvantages of employing enzymes in comparison with chemical synthesis routes are given in Table 1.2 below.

Liese and Filho (1999) concluded that the future of biocatalysis is arguably through integration with chemical synthesis routes in multi-step catalysis. Kim *et al.* (2002) demonstrated the ability of biocatalysis to integrate with chemistry when they coupled the use of metal catalysis with lipase enzymes for the dynamic kinetic resolution of functionalised alcohols. Whilst overall product yields appeared to be low (13% overall), Mazzini *et al.* (1997) demonstrated the potential of Baeyer-Villiger biocatalysis to integrate with chemical synthesis, when a biotransformation formed the key step in a seven-step enantioselective synthesis of (R)-(-)-Baclofen.

Advantages	Disadvantages
Mild operating conditions, typically 37°C, pH 7 and 1 atm	Low volumetric productivities are common
High chemo-, regio-, enantio- and diasterioselectivity.	Absolute substrate specificity may make the process inflexible
Potential to perform complex reactions with no equivalent in traditional chemistry, e.g. introduction of functional groups at otherwise unreactive sites.	Possibility of unwanted side reactions with whole cell biocatalysts
Potential for a low environmental impact process	Biocatalysts may show poor operational stability, often not being re-useable.
Use of an aqueous reaction medium reducing the need for organic solvents	Bulk removal of water, caused by low product concentrations, may be a problem in downstream processing
Fewer and sometimes cheaper reagents	Biocatalyst production may be expensive
Potential for fewer reaction by-products due to biocatalyst selectivity	Reaction time tends to be long
Potential avoidance of protection and de-protection steps	

Table 1.2: The potential advantages and disadvantages of enzymes compared to traditional chemical synthesis (adapted from Cheetham, 1994; Tramper, 1996 and Lai and Lee, 2002).

1.1.5 Oxygenase enzymes

As this project concentrates on the oxygenase enzymes they merit separate mention. According to a review of biotransformations using oxygenases by Li *et al.* (2002) there are three main reasons why oxygenase enzymes are important:

1. Their chemical counterparts either do not exist or do not have the required regio- or stereoselectivity.

2. They use oxygen as a cheap and environmentally friendly oxidant in contrast to the toxic chemical oxidants.
3. They can be used to modify natural products with biological activity or to prepare chiral building blocks for industrially relevant targets.

Oxygenases are of interest due to the high level of chemo-, regio- and stereospecificity achievable, and thus they are a typical example of why enzymes themselves are of commercial interest (Table 1.2).

Oxygenases have been classified into two groups (Kelly *et al.*, 1998):

1. Monooxygenases which catalyse the insertion of one oxygen atom from molecular oxygen into the substrate, the other undergoing reduction to H_2O.
2. Dioxygenases which catalyse the insertion of both oxygen atoms into the substrate.

The former are more common and are the subject of the research in this project.

Monooxygenase enzymes employ a range of prosthetic groups, including:

- Iron (e.g. Cytochrome P450)
- Copper (e.g. dopamine β-hydroxylase)
- Flavins and cofactors nicotinamide adenine dinucleotide phosphate (NAD(P)H) (e.g. cyclohexanone monooxygenase (CHMO))

Monooxygenases that employ flavins, of which there are three found in nature (riboflavin, flavin mononucleotide and flavin adenine dinucleotide (FAD)), are called flavoenzymes.

Oxygenase reactions tend to exhibit low volumetric productivity, therefore to be economically feasible the products have to be high value. One of the few commercial applications of oxygenase enzymes to date has been in the production of steroids. This is demonstrated by the K_{cat} values of NAD(P)H-dependent oxygenases (Table 1.3 shows the K_{cat} values of CHMO and CYP2C9), which are relatively low (0.2 s^{-1} to 75 s^{-1}) in comparison to hydrolases (which often have K_{cat} values exceeding 100,000 s^{-1}), which find relatively wide industrial application (section 1.1.1) (Duetz *et al.*, 2001).

Enzyme	Substrate	K_{cat} (s^{-1})
CYP450 (CYP2C9)	Diclofenac	0.3
CHMO	Cyclohexanone	17

Table 1.3: Comparative K_{cat} values for CHMO and a cytochrome P450 (CYP450) enzyme (Duetz *et al.*, 2001).

Whilst at lower oxygenase activity levels (~100 U/g (dry weight)) NAD(P)H regeneration rates may not be expected to limit enzyme activity, at higher levels (1000-4500 U/g (dry weight)) they may become limiting (Duetz *et al.*, 2001).

The integration of oxygenases into synthetic processes for fine chemicals (Duetz *et al.*, 2001; van Beilen *et al.*, 2003) faces several hurdles in comparison with easy-to-use enzymes such as isomerases, lyases and hydrolases:

- Enzyme instability
- Vulnerability of the microbial hosts cells to organic substrates/products.
- The special skills required for the handling of these biocatalysts.
- They consist of multiple components, some of which are membrane-bound or require expensive cofactors such as NAD(P)H
- The desired regio-, chemo- and enantiospecific reactions require elaborate screening procedures.
- They often require complex reaction media such as bi-phasic systems, as the hydrocarbon products are often not water-soluble.

The current industrial use of oxygenases and other enzymes are discussed in Section 1.1.9 below.

1.1.6 Comparison of enzyme hosts

Whilst whole cell biocatalysis is possible with microbial cells, with plant and animal cells, enzyme isolation and purification is usually unavoidable due to low relative enzyme concentrations, difficulty of growth and current relatively poor understanding (Oksman-Caldentey and Inze, 2004). For similar reasons, plant and animal cells are usually neither grown for enzyme production, nor genetically modified for increased

enzyme production in whole cells (Tramper, 1996). Sicard *et al.* (2005) noted that most enzymes of industrial interest are currently isolated from microbial sources or from genetic libraries, however enzymes chymotrypsin and papain are noteable exceptions.

The reason for the increased interest in microbial cells is the advantages they have over plant and animal cells (Roberts 1995):

- The small size of the cells, particularly bacterial, means there is a high specific surface area and they are able to grow relatively quickly and avoid contamination; and
- Microbial cells are easier to grow due to the cell wall structure, which increases their mechanical stability.

There are standard cell hosts that are used for the genetic modification of microbial cells lines, generally selected for their low cost, speed of growth, relatively well known genetics and thus their ease of manipulation, non-toxic nature, ready availability and industrial relevance. The two different subclasses of microbial cells are:

- Yeasts, where *Saccharomyces cerevisiae* (Baker's Yeast) is commonly used (Jiang and Morgan, 2004; Ward 1995)
- Bacteria, where *Escherichia coli* has become the workhorse of molecular biology and biotechnology (Watts *et al.*, 2005).

In contrast with microbial cell hosts, plants, of which there are over four hundred thousand higher species, have shown a rich source of secondary metabolites with over one hundred thousand natural products being extracted (Watts *et al.*, 2005). However due to the costly nature of extraction from plants, utilising plant-based biosynthetic pathways for biocatalysis represents an attractive potential alternative (Oksman-Caldentey and Inze, 2004).

1.1.7 Drivers of biocatalyst development

The utilisation of enzymes is a rapidly emerging field as a consequence of the great advances made in a combination of discovery technology areas (Fessner, 2003):

Structural biology – Whilst understanding of the relationship between protein structure and function for enzymes is still lacking, advances in crystallography and protein analysis have derived more than nineteen thousand experimentally determined high resolution protein structures. Driven by the publication of the crystal structure of phenylacetone monooxygenase, Bocola *et al.* (2005) were able to mutate the active site enhancing the range of substrates accepted, and enantioselectivity of, this BVMO enzyme.

Molecular biology developments have proved crucial for the production of novel biocatalysts with high catalytic activity and stability (Affholter, 1999; Ager *et al.*, 2000). The following key techniques have been developed:

1. Molecular cloning and gene amplification. For example Doig *et al.* (2001a) cloned an enzyme (CHMO) from the wild type host (a class 2 pathogen) into *E. coli* and thus successfully removed a detrimental side reaction, whilst facilitating over-expression of the enzyme. (For a recent review of strain improvement by genetic engineering, see Chaing, 2004).
2. Random and site-directed mutagenesis, i.e. directed evolution via the Polymerase Chain Reaction (PCR). For example Dufour *et al.* (1998) used site directed mutagenesis to engineer nitrile-hydrolase activity into papain giving rise to a 4000-fold increase in activity compared with the wild-type papain, whilst simultaneously producing a catalyst that was stable, not susceptible to substrate inhibition and could operate in organic solvents. Chodorge *et al.* (2005) employed only two weeks of directed evolution to yield a seven-fold improvement to the thermostability of lipase B. (The following recent review articles on the potential of directed evolution have been published - Otten and Quax, 2005; Hibbert *et al.*, 2005; Eijsink *et al.*, 2005)

Microbial genomics has lead to the sequencing of around five hundred genomes. The unravelling and publication of the genome DNA sequences of industrially used microbial production strains facilitates further strain improvement and development through metabolic engineering. Fraaije *et al.* (2005) identified a Baeyer-Villiger monooxygenase (BVMO) genome from *Thermobifida fusca* and successfully expressed it in *E. coli* to create a novel biocatalyst.

Synthetic biotechnology has enabled novel assay technology for high sensitivity and specificity to be coupled with technological advances in high throughput screening (Soumillion and Fastrez, 2001) and laboratory automation. This has allowed biocatalysts with desired activity and stereospecificity to be selected from large libraries (Stahl *et al.*, 2000), see **biological libraries**.

Bioprocess engineering – Advances in new reaction media, i.e. biphasic systems (Van Sonsbeek *et al.*, 1993; Schmid *et al.*, 1998a; Shin and Kim, 1999), ionic liquids (Yang and Pan, 2005) and supercritical carbon dioxide (Matsuda *et al.*, 2005) and pure organic solvents (Schmid et al., 1998a), have offered alternatives that expand the technical boundaries for the utilization of enzymes. The combination of chemical and biochemical steps in aqueous reactions, without the need for substrate functional-group protection, has potentially improved the applicability of biocatalysis to pharmaceutical production (Kim *et al.*, 2002). The use of substrate feeding (Doig *et al.*, 2002b) and adsorbent resins (Hilker *et al.*, 2004b) has begun to address the problem of substrate and product inhibition, and modelling has increased the understanding of biocatalytic processes (Chen *et al.*, 2002).

Other areas of consequence:

Regulation – Increasing safety regulations and environmental awareness have fostered a need for "green chemistry", which in turn is fostering the acceptance of biocatalytic alternatives to chemical synthesis. This is discussed further in section 1.5.

Biological libraries – the creation of large, diverse enzyme libraries available for screening (Soumillion and Fastrez, 2001) will significantly reduce bioprocess

development lead times and will broaden the scope of opportunities for biocatalytic process.

Schulz and Wubbolts (1999) draw the conclusion that these technologies are changing biocatalysis from an enabling tool to a lowest cost approach. The commercial interest in biocatalysis certainly appears to be increasing; among patent applications in the last decade, the fraction related to biotechnology has increased from 31% to 41% (Fessner, 2003).

1.1.8 Comparison of isolated enzyme, whole cells and immobilised whole cells

There are two main types of biocatalyst commonly employed; isolated enzyme and whole cell systems, both of which have their own merits. Due to the relative instability of isolated enzymes, they are usually immobilised prior to industrial use. Of the twelve most useful commercial bioconversions discussed by Cheetham (1994), about half contain immobilised enzymes in continuous bioreactors. The predominant reason for this is that they tend to produce high volume, low value products, meaning that any process improvement is likely to produce large cost savings, thus the increased amount of research and optimisation required for such systems is justified. For a recent review of the commercial application of immobilised enzymes and biocatalysts from microbial, plant and animal sources see End and Schoning (2004).

The immobilisation of whole cells by comparison is less common. A comparison of the different systems is shown in table 1.4. Whole cells are generally difficult to separate from the reaction broth at scale and are thus often used as a reagent rather than a catalyst, however the enzyme tends to be more stable and can convert a wider range of substrates because of this. Immobilisation of whole cells can increase the stability of the enzyme and facilitate re-use, however the increased mass transfer resistances of such systems results in reduced enzyme activities. Immobilised enzymes are more stable due to the removal of unwanted side reactions and can enable more concentrated substrate and product solutions to be used, however the process of immobilisation can be expensive.

<table>
<tr><th></th><th>Whole cells</th><th>Immobilised whole cells</th><th>Immobilised isolated enzymes</th></tr>
<tr><td>Enzyme Activity</td><td>Higher than immobilised whole cells.</td><td>Lower than with free whole cells due to increased mass transfer resistances.</td><td>Can be higher than whole cells if mass transfer of substrate/product or oxygen is the rate limiting step.</td></tr>
<tr><td>Separation and re-use</td><td>Separation often difficult at large scale. Sometimes biocatalyst effectively becomes a stoichiometric reagent.</td><td colspan="2">Product purity and subsequent separation and purification are simpler than with free whole cells.</td></tr>
<tr><td>Volumetric productivity</td><td>Often higher than immobilised whole cells due to higher reaction rates.</td><td>Can be higher or lower than free whole cells depending upon trade off between reaction rate reduction and increased stability allowing increased concentrations.</td><td>Often high compared to whole free cells as more concentrated solutions can be used.</td></tr>
<tr><td rowspan="2">Stability</td><td colspan="2">Enzymes are more stable in cells than when isolated, often enabling them to convert a wider range of substrates.</td><td>Remove potentially unwanted side reactions.</td></tr>
<tr><td></td><td>Stability of intra-cellular enzymes can be increased by immobilisation, allowing improved activity longevity.</td><td>Are usually immobilised to give stability, often allowing multiple re-use.</td></tr>
<tr><td rowspan="2">Other features</td><td>Cheap to manufacture, with reduced set-up times compared to isolated enzymes or immobilised whole cells.</td><td colspan="2">Biocatalysts can be fixed in place enabling multi-step conversions, including integration with chemical steps.</td></tr>
<tr><td>Compared with immobilised whole cells, oxygen limitation is less problematic.</td><td colspan="2">Can be used in organic solvents (whole cells need biphasic mixtures to maintain the required aqueous phase)</td></tr>
</table>

Table 1.4: A comparison of isolated enzymes, whole cells and immobilised whole cells, the most important to this project being coloured blue. Adapted from Endo *et al.* (2001), Buque *et al.* (2002) and Jianlong *et al.* (1999).

There are four main reasons, which particularly hold true for oxygenases, why whole cells should be used rather than isolated enzymes (Duetz *et al.*, 2001):

1. If the presence of other enzymes has no negative impact, economically the cost of isolating and purifying the enzymes can be significant. Furthermore the presence of the cell walls protects the enzymes from shear forces, which can damage enzyme activity in stirred bioreactors.
2. The membrane bound nature of most enzymes can lead to nearly complete loss of activity if removed. From research published to date this clearly doesn't hold for the CHMO isolated enzyme.
3. Cascades of enzyme reactions may be too complicated to perform outside the cell due to the number of enzymes, cofactors and substrates involved.
4. The stoichiometric consumption of cofactors NAD(P)H or NADH, and their associated prosthetic groups FAD and FMN during reactions make whole cells attractive.

A significant weakness of isolated oxygenases is that they require cofactors and thus to operate effectively the cofactors must be recycled. Three industrial methods of cofactor recycling have been demonstrated (Rozzell, 1999):

- Use of a coupled reaction. For example NAD(P) was converted to NAD(P)H using hydrogen to regenerate horse-liver alcohol-dehydrogenase (HLADH) by Andersson *et al.* (1998). Such a technique is employed in the recycling of NAD(P)H for the cytochrome P450 catalysed metabolism of drugs, studied in chapter 3.
- Use of a macro-molecularised cofactor in a membrane reactor (Kragl *et al.*, 1996)
- The use of whole cells and an energy source. This method as is typically used in whole cell biocatalysis and is employed in the cyclohexanone monooxygenase biocatalysis studied in chapter 2.

More recent research has yielded another promising alternative; the electrochemical regeneration of cofactors. This uses electrons as cheap redox equivalents and has the benefit of allowing the full catalytic activity of the enzymes to be used. Figure 1.1

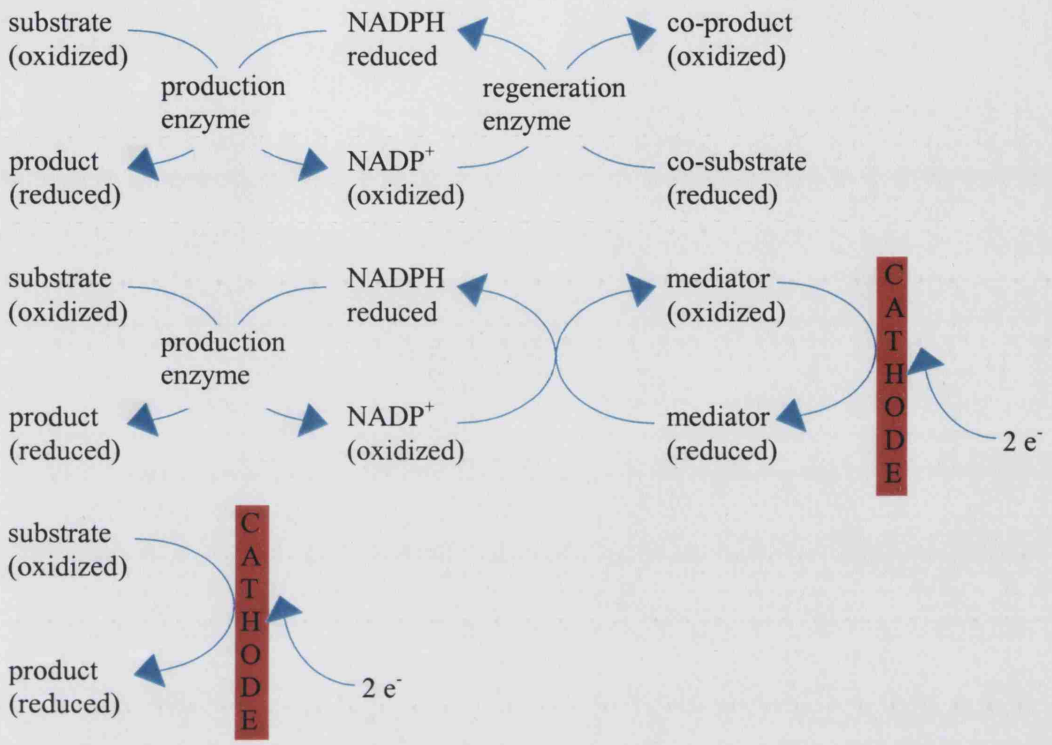


Figure 1.1: Comparison of chemical and electrochemical cofactor recycling methods.

Top: regeneration enzyme with sacrificial substrate.

Middle: Electrochemical regeneration with catalytic of mediator

Bottom: Direct electrochemical regeneration.

Adapted from Liese (2005).

gives a comparison between the biochemical and electrochemical cofactor recycling methods.

The efficient recycling of NAD(P)H, as is required for the CHMO oxidation studied in Chapter 2, has been reported using water-soluble polyethylene glycol bound Cp*Rh(bipyridyl)-complexes as mediators in continuously operated bioreactors (Hollmann *et al.*, 2001; Hollmann *et al.*, 2002; Schmid *et al.*, 2001). A recent review of electrochemical cofactor regeneration has been written by Steckhan *et al.* (2001).

The problem of cofactor recycling is one of the predominant reasons for selecting whole cell biocatalysts rather than isolated enzymes.

1.1.9 Whole cell immobilisation

Improving methods of integrating chemical synthesis routes with biocatalytic synthesis is a key factor in the increased industrial application of biocatalysis. Due to the ease of separation and handling, and their potential for increased enzyme stability, immobilised cells could gain increasing industrial significance.

Cells are generally grown until they are at optimal conditions for free cell activity prior to being immobilised. Different methods of enzyme immobilisation are shown in Figure 1.2. Whilst any of these methods could potentially be employed for the immobilisation of whole cells, of the four different types, only the first three of which were shown to be feasible for whole cell immobilisation by Roberts (1995):

1. Entrapment in a polymer matrix, e.g. alginate, polyacrylamide, κ-carrageenan.
2. Surface adsorption onto a water-insoluble solid support, e.g. ion exchange resins
3. Covalent attachment to water-insoluble solid supports, e.g. cellulose
4. Chemical cross linking with bi-functional agents, e.g. toluene diisocyanate

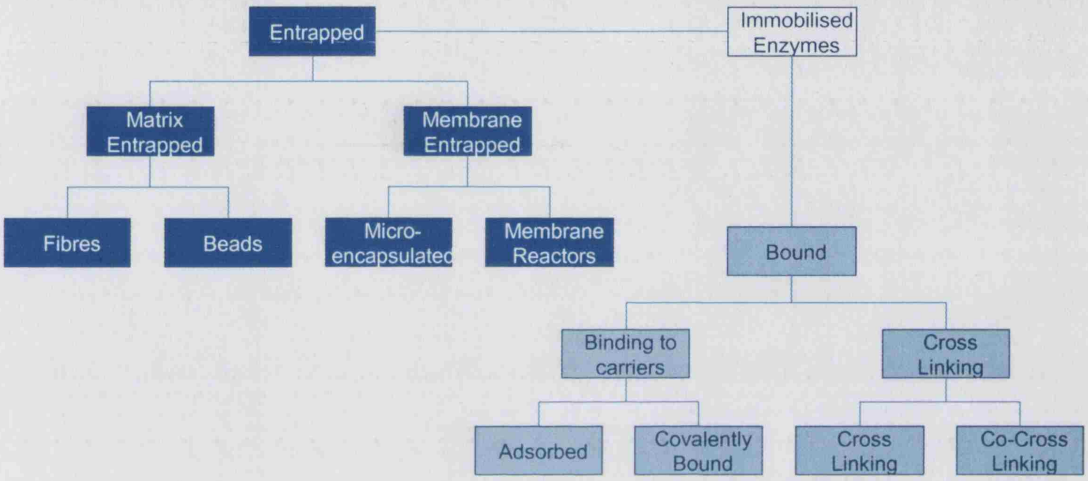


Figure 1.2: Methods of enzyme immobilisation. Adapted from Nedovic and Willaert (2003).

Kallenburg *et al.* (2005) review a wide range of Penicillin G Acylase enzyme immobilisation methods and concluded that no single best method for immobilisation could be recommended. They noted that mass transfer limitation, which is closely linked to particle size, is the main limiting factor to further process improvements. From this the whole cell immobilisation method which facilitates the use of higher concentrations of substrate and product, provides the highest volumetric productivity and enzyme stability would appear to be the most beneficial.

Immobilisation can be employed to prevent unwanted side reactions, as often observed with wild type cells, the rate limiting diffusion taken advantage of to alter the concentration levels to which cells are exposed. Carballeira *et al.* (2004) found that immobilized cells (*Geotrichum candidum* NCYC49) could be used to alter the yield of *e*-caprolactone from the oxidation of cyclohexanone by supplying high amounts of oxygen which preferentially converted the cyclohexanone to the lactone (90-100% yield) rather than to cyclohexanol (none detected).

Immobilization by entrapment in biopolymer gels, such as alginate is a well established technique. It is simple to perform, readily available, does not require functionalisation of the support, has a high affinity for aqueous solutions and is regarded as biocompatible due to the mild reaction conditions (Buque *et al.*, 2002; Kallenberg *et al.*, 2005). Calcium alginate has been a promising method for microbial degradation of toxic substances and has been used since 1975 (Chung *et al.*, 2003) and has become the most widely used technique for immobilizing living cells (Strand *et al.*, 2000).

The immobilisation of cells in alginate beads has been shown to protect the bacteria against the toxicity of phenol (Heipieper *et al.*, 1991). However, one of the disadvantages of using cells entrapped in calcium alginate gel beads, like in many other immobilisation techniques, is the mass transfer limitations that often result (Kallenburg *et al.*, 2005). An important advantage of immobilisation in alginate gels over other immobilisation methods is its low toxicity, making it suitable for application in the pharmaceutical industry (Drury *et al.*, 2004). Fernandes *et al.* (2002) found that calcium alginate immobilised cells gave a higher specific enzyme activity

than k-carrageenan and celite immobilised cells, with the activity seen being greater than that of the free cells.

Whilst the scale-up of bead production is thought to be of concern, *E. coli* cells displaying high aspartase activity which have been immobilised in κ-carrageenan, have been industrially employed in Japan since 1973 (Leuenberger, 1990). As such this is likely to represent an area where industrial research has been performed but remains unpublished.

For immobilised systems, Thiele modulus (a dimensionless number) is used as a measure of whether immobilised enzyme reactions are diffusion or reaction rate limited:

$$\phi = r_0 \sqrt{\frac{k}{D_e}}$$

Where r_0 is the radius of the bead (cm), D_e is the effective diffusivity within the bead and k is the first order rate constant.

However processes employing immobilised living cells are more complicated and thus the measure of Thiele modulus may give unreliable interpretation of the performance of such biocatalysts (Beschkov and Velizarov, 2000). Mota *et al.* (2002) modelled diffusivity in a gel immobilised system and concluded that whilst their model was effective for some published data, there is an absence of reliable information associated with diffusivity in immobilised systems, and much work needs to be done. Zhao and DeLancey (2000) generated a model based on nineteen reported reactions, which they claim shows some predictive capability, but is not simple to apply. Laca *et al.* (2000) give an excellent overview of the many different models used for diffusivity and reaction rate and the complications that multi-parameter immobilisation poses. Such modelling is outside the scope of this project and thus the diffusivity in the system will not be assessed in terms of the Thiele modulus and effective diffusivity, but will be considered from its effect on limiting the apparent rate of reaction.

As the free cell reaction rate is thought to be mass transfer limited, rather than reaction rate limited (from comparison of the reaction rate of isolated enzyme and the free cell

systems) then a measure comparing free cells and immobilised cells to the isolated enzyme reaction rate and also comparing immobilised cells against free cells will be used. The catalyst effectiveness factor as described by Liu *et al.* (2005):

$$\eta = \frac{\text{Rate with diffusion resistance}}{\text{Rate without diffusion resistance}} = \frac{\text{Observed rate}}{\text{Intrinsic rate}}$$

This will be developed further in section 2.5.

1.1.10 Industrial application and regulation

Due to their commercial availability and the relative ease with which they can be used at scale, the most commonly used enzymes at industrial scale are:

- Lipases (EC 3.1.1), commonly used in yogurt, cheese fermentation, baking, laundry detergents and as biocatalysts converting vegetable oil into fuel (Matsumae *et al*, 1993; Anderson *et al*, 1998; Ban *et al*, 2001).
- Amidases (EC 3.5.1.4) (Sonke *et al*, 1999)
- Dehalogenases (Ladner and Ditrich, 1999)

Conversely, although some enzymes have great potential as their products are impossible to synthesize chemically, their commercial implementation is less common. Typical such enzymes include:

- Decarboxylases (Ward and Singh, 2000)
- Asymmetric C-C bond formation (Turner, 2000)
- Oxidoreductive boconversions, industrial uses to date being show in Table 1.5 below: where alcohol dehydrogenase has been used for the chiral synthesis of chemicals and hoseradish peroxidise used as a laundry and wood pulp bleach (Holland and Weber, 2000; Duetz *et al*, 2001; Boyd *et al*, 2001)

Enzyme	Source	Application
Alcohol dehydrogenase	*Saccharomyces cerevisiae, Thermoanarobium brockii*	Chiral synthesis of chemicals
Amino acid oxidase	Porcine kidney, snake venom	Chiral resolution of racemic amino acid mixtures
Catalase	*Aspergillus niger*	desugaring of eggs
Chloroperoxidase	Algae, bacteria, fungi, mammalian tissues	Steroid synthesis
Peroxidase	Horseradish	Laundry and wood pulp bleaches

Table 1.5 – Industrially applied oxidoreductases.

The key data, which must be investigated to assess the economic feasibility of a bioconversion, are shown in Figure 1.2.

Generally there have been three classes of biocatalysis applied in industry (Burk, 2003):

1. Single step transformations catalysed by purified or immobilised enzymes, e.g. Aspartame (Holland Sweetener company)
2. Whole cell biocatalytic reactions, often the most economically method of employing enzymes, e.g. acrylamide, L-carnitine.
3. Multiple enzyme processes, generally via cell culture fermentation.

As discussed in section 1.1.2, one of the predominant reasons why enzymes are industrially employed is due to the stereospecificity that they yield. Table 1.6 gives an example of enzymes applied commercially for that reason.

Product (Use)	Use	Scale (tpa)
aspartame	sweetener	2000
L-aspartic acid	sweetener component	Not available
L-carnitine	slimming aid	300
S-2-chloropropanoic acid	herbicide precursor	2000
D-p-hydroxyphenylglycine	antibiotic component	> 1000
S-1-methoxy-2-aminopropane	agrochemical intermediate	multiple
L-tert leucine	chiral intermediate	1-2

Table 1.6: Some commercial, industrial-scale biocatalytic processes, implemented due to the increased stereoselectivity offered by biocatalysis. Adapted from Liese *et al.* (1999).

An increasing global awareness of the environmental impact of industrial processes is leading to legislation to lessen this impact (Olguin, 2000). The result is that biocatalytic synthesis routes are again being considered more favourably as they can often demonstrate significant environmental advantages over an equivalent chemical process (Scragg, 1999).

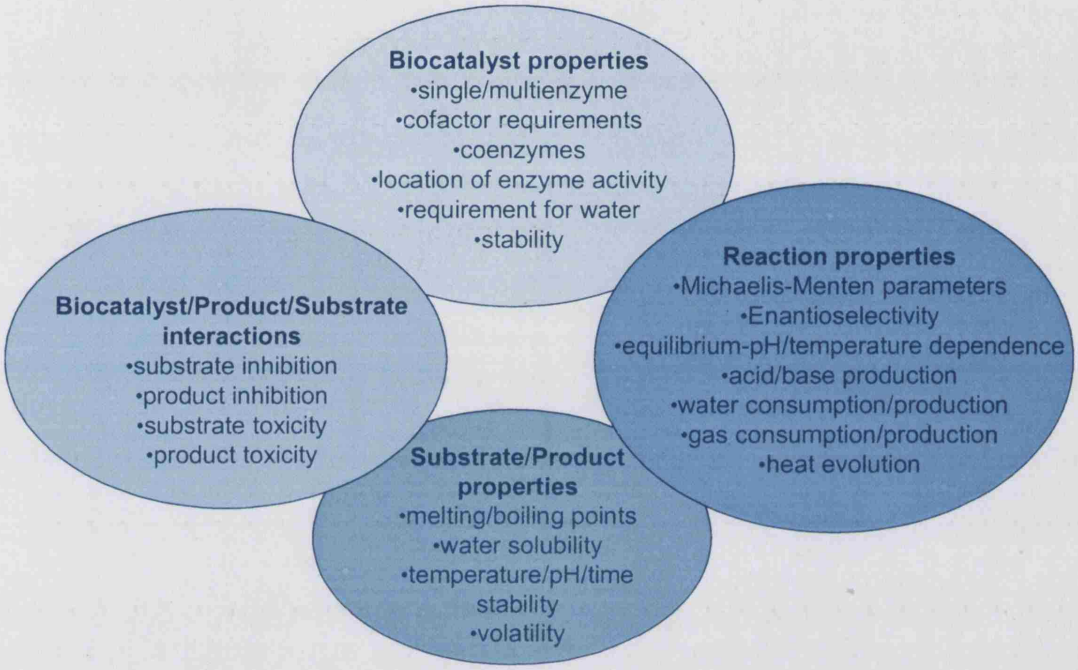


Figure 1.3: Key data required to fully characterise a bioconversion and define the bioprocess design constraints. Adapted from Woodley and Lilly (1994).

1.2 CHMO

1.2.1 Introduction to CHMO

The Baeyer-Villiger reaction is defined as the conversion of a ketone to an ester by a hydroperoxide (Kelly, 2000). The usefulness and generality is such that a major review by Krow (1993) was 547 pages long with 420 pages of examples.

Baeyer-Villiger monooxygenases (BVMOs) are a type of monooxygenase that mediate the Baeyer-Villiger type nucleophilic oxygnenation of linear or cyclic ketones, yielding the corresponding esters or lactones. They use a flavin coenzyme (FAD) as a cofactor and NAD(P)H as a reductant (Willetts, 1997).

The Baeyer-Villiger reaction (Figure 1.4) can be achieved biochemically with a BVMO enzyme or chemically, conventionally by employing an oxidant such as peracetic acid (Coleman *et al.*, 1997), m-chloroperbenzoic acid, peroxybenzoic acid and hydrogen peroxide (Renz and Meunier, 1999). The main problem with the conventional chemical route is that these oxidants are often toxic, explosive, often lose stereospecificity, generate undesired by-products and almost always show low enantioselectivity (Renz and Meunier, 1999; Hunt and Grieco, 2000).

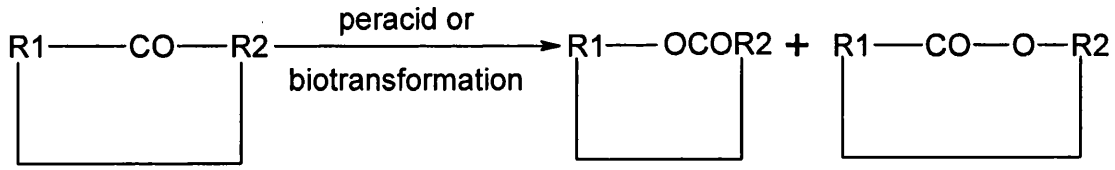


Figure 1.4: The Baeyer-Villiger reaction

Due to the interest seen in the biocatalytic route, chemistry has attempted to keep pace with developments with some limited success. Of the three reasons proposed by Li *et al.* (2002), section 1.1.5, for the importance of oxygenases, the first (poor regio- or stereoselectivity of chemical catalysts) has been challenged with moderate success on two fronts:

- By chemical oxidation using transition metal catalysts, where a good enantiomeric excess (*ee)* was achieved on selected substrates, the maximum reported being an

87% *ee* on 3-phenyl cyclobutanone (Strukul, 1998; Bull *et al.*, 1999; Corma *et al.*, 2001; Bolm *et al.*, 2001; Watanabe *et al.*, 2002). These catalysts are, however, toxic, require relatively high operating temperatures and pressures, and often necessitate the use of multiple, difficult chemical protection steps (Sheldon, 2000).

- By metal-free organocatalytic methods where flavin based catalysts are designed. These have the potential advantage over enzymes of being more stable (Murahashi *et al.*, 2002), however the average stereospecificity achieved was only an *ee* of 60%, with the maximum being 74%.

Beller (2004), in his overview of oxidation chemistry, stresses the need for greener waste avoiding chemical synthesis routes, and stresses the importance of H_2O_2 or O_2 as oxidants as their only by-product is water. Chemical routes using H_2O_2 and O_2 have been discussed above, however biocatalytic synthesis routes which employ molecular oxygen, also operate at milder operating conditions, and thus, on an environmental basis, oxygenase enzymes have been argued to be a greener alternative to their chemical counterparts (Mihovilovic *et al.*, 2002)

Baeyer-Villigerases are monooxygenase enzymes that typically require the use of a cofactor. As of 2000, only five of these had been purified and tested with a selection of substrates (Table 1.7).

Name –mono-oxygenase (MO)	**Abbreviation**	**Cofactor**
Cyclohexanone-	CHMO	NAD(P)H
Cyclopentanone-	CPMO	NAD(P)H
2,5-diketocamphane 1,2-	2,5-DKCMO*	$NADP^+$
3,6-diketocamphane 1,6-	3,6-DKCMO*	$NADP^+$
2-oxo-Δ^3-4,5,5-trimethylcyclo-pentenylacetic acid	MO2	NAD(P)H

*A mixture of these two enzymes was originally named MO1

Table 1.7: The five studied Baeyer-Villigerases. Adapted from Kelly (2000).

CHMO is the best characterized BVMO (Doig *et al.*, 2001) and forms the basis of the first part of this project. A complexity with some of the other enzymes is that the wild type cell host *Pseudomonas putida* NCIMB10007, expresses all of the latter three enzymes (Gagnon *et al.*, 1994) making them more difficult to isolate. However, a potentially useful aspect of 2,5- and 3,6-DKCMO BVMOs is that they proceed with the opposite stereospecificity to the other enzymes (Kelly, 2000).

1.2.2 Research findings to date

The CHMO catalysed synthesis of enantiopure lactones from the racemic ketone, bicyclo[3,2,0]hept-6-en-2-one forms the backbone of this study into the whole cell immobilisation and inhibition seen in Chapter 2. This reaction scheme can be found in Figure 1.5.

This reaction has been the focus of considerable academic research, and as such represents a good model reaction for further study. The predominant reasons for the selection of this enzyme and substrate combination for study are:

A. The enzyme

- CHMO is an oxygenase enzyme which has shown great potential for a synthesis route to optically pure lactones, many of which cannot be accessed chemically.
- The enzyme is readily cloned into alternative hosts (see section 1.2.2.1). This is important as the wild type host cell (*Acinetobacter calcoaceticus* NCIMB 9871) has several drawbacks:
    1. It is a class 2 pathogen,
    2. Grows on a toxic carbon source (cyclohexanol),
    3. Exhibits low CHMO activity (typically 20-25 U (g dwt)$^{-1}$, Trudgill, 1990), and
    4. Contains a lactone hydrolase which can further metabolise the product and cause down stream processing problems unless the CHMO is isolated or it is inhibited, e.g. by tetraethyl-pyrophosphate.

(Carnell *et al.*, 1991; Alphand and Furstoss, 1992b; Konigsberger and Griengl, 1994; Barclay *et al.*, 1997, Doig *et al.*, 2001a)

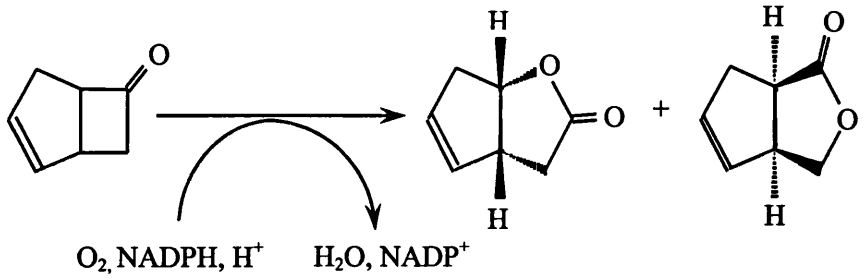


Figure 1.5: Reaction scheme of bicyclo[3,2,0]hept-6-en-2-one to its two lactone regio-isomers, (-)-(1S,5R)-2-oxa-bicyclo[3,3,0]oct-6-en-2-one and (-)-(1R,5S)-3-oxa-bicyclo[3,3,0]oct-6-en-2-one.

B. The substrates and products

- The reaction is representative of those often seen with oxygenase enzymes due to:
    - The inhibitory and, at higher concentrations, toxic nature of the substrate and products.
    - The high oxygen requirement which, along with the diffusion rate of substrate and product may be rate limiting.
- Due to the high degree of enantiopurity of the products they represent interesting potential pharmaceutical intermediates, odorant and flavouring constituents (Kelly, 2000).
- The substrate and products have sufficient aqueous solubility to enable them to be used in water-rich biocatalytic reactions.

An overview of some of the constraints on this reaction and potential methods of overcoming them are diagrammatically represented in figure 1.6. These and other recent research is discussed below.

1.2.2.1 Biocatalyst host

The practical challenges for the development of oxygenase biocatalysts include the development of strains with (Duetz *et al.*, 2001):

- Low endogenous oxygen respiration rates
- High NAD(P)H regeneration rates
- Long oxygenase half-life
- Low sensitivity of the host and enzyme to organic solvents and product inhibition

As mentioned in section 1.2.2, the wild type host contains numerous negative factors, therefore attempts have been made, with varying success, to clone the CHMO enzyme into alternative hosts:

A number of whole cell yeast biocatalysts expressing CHMO have been developed (Stewart *et al.*, 1996; Kayser, 1999). Although the catalytic activity of these hosts had been proven, the presence of alcohol dehydrogenase appeared to competitively reduce

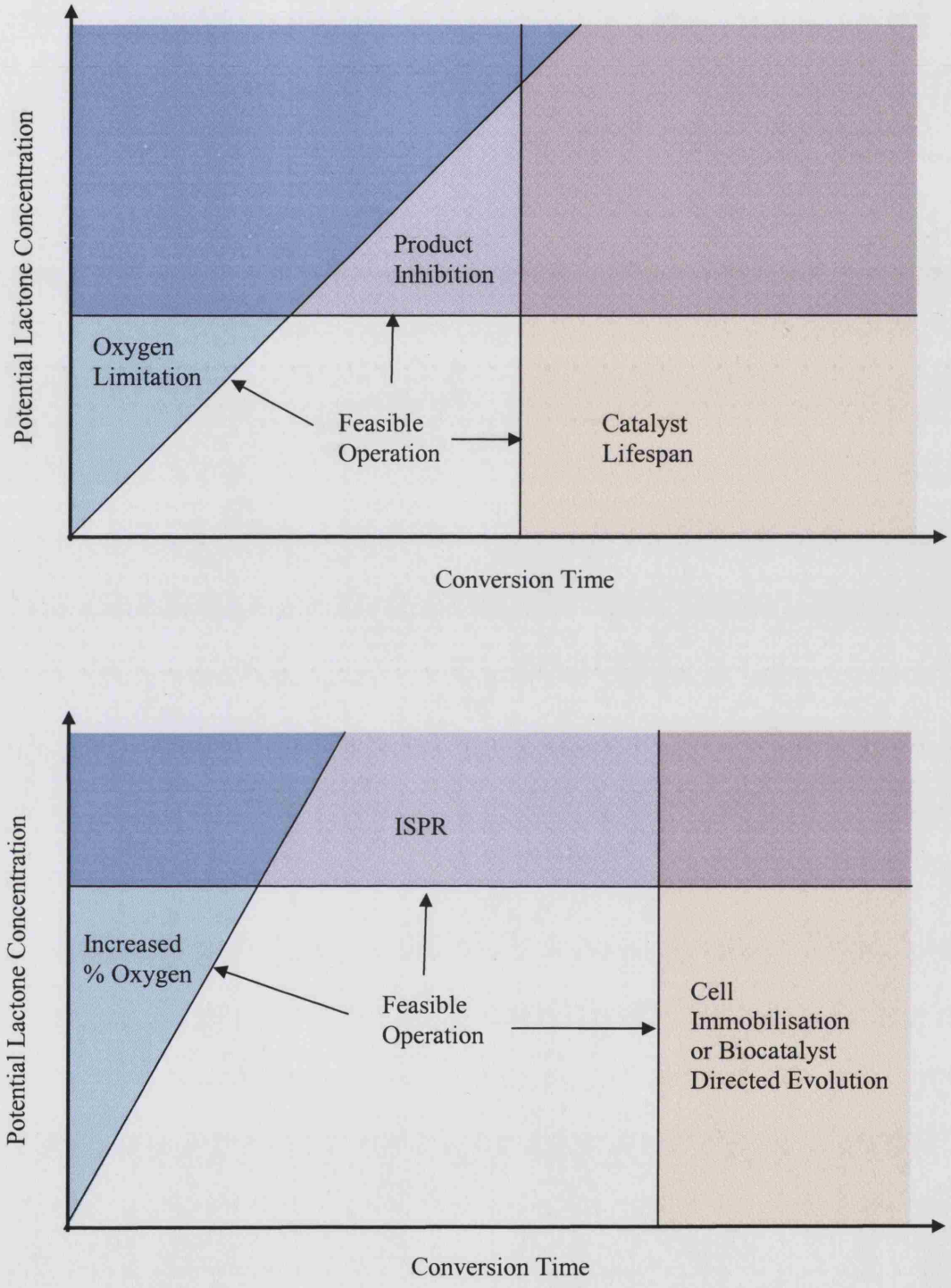


Figure 1.6: Overview of the constraints on CHMO biocatalysis (top) and potential methods of overcoming them (bottom). Based on the windows of operation method of process mapping developed by Chen *et al.* (2002).

the ketone. To optimize turnover rates, increase biomass efficiency and decrease the probability of undesired side reactions the CHMO was cloned into *E. coli* (strain BL21 (DE3)) and was then developed under the control of an isopropylthio-β-D-galactoside (IPTG) inducible promoter (Chen *et al.*, 1999). In this strain, CHMO represented 20% of the cellular protein, over a 10-fold improvement compared to the yeasts.

At UCL a microbial strain expressing CHMO in an L-arabinose inducible vector has been constructed; *E. coli* TOP 10 [pQR239] expressing CHMO at 500 to 600 U/g (DCW) (U = μmol/min enzyme activity). The construction of this strain and subsequent large scale fermentation was reported by Doig *et al.* (2001a). The primary advantage of the TOP10 strain over that developed by Chen *et al.* (1999) is the low cost nature of the inducer L-arabinose in comparison to IPTG.

E. coli does not exhibit any of the negative factors associated with the wild type host (1 to 4, section 1.2.2). It is also well characterized and can be simply grown (Watts *et al.*, 2005). Potential weaknesses seen with this *E. coli* strain are that the enzyme is only stable in use for around sixteen hours (see sections 1.2.2.3 & 1.2.2.5), and substrates and products are still inhibitory at relatively low levels.

In an attempt to lessen the impact of these weaknesses, CHMO was recently cloned into *Pseudomonas putida* AC34 pQR266 at UCL, early indications of which are a lessened impact of inhibition, with the reaction rate being only slightly reduced (Doig *et al.*, in press). Whilst the stability of the enzyme in this host has still to be rigorously tested, due to the increased mechanical stability of *P. putida* this host could represent a reasonable process improvement in terms of biocatalyst yield.

1.2.2.2 Substrate inhibition

Doig *et al.* (2003) found that the substrate bicyclo[3,2,0]hept-6-en-2-one (reaction scheme given in Figure 1.5) concentration became inhibitory in the wild type host and with the *E. coli* TOP10 strain at around 1g/L. This level of inhibition is also seen with

the yeast strains constructed by Kayser *et al.* (1997). A more in-depth substrate inhibition profile was studied by Doig *et al.* (2003) on *E. coli* TOP10 and the optimal operating substrate range of 0.2-0.4g/L was described.

To prevent inhibitory substrate concentrations, a simple fed-batch biocatalysis method has been adopted (Doig *et al.*, 2002a) and used at scale. A complicating factor with such a seemingly simple strategy is the time taken to perform GC sample analysis, making control difficult and feeding rates less responsive. To overcome this potential drawback, at-line monitoring by near infra-red spectrometry has been successfully employed (Bird *et al.*, 2002), reducing the monitoring time from around 35 minutes to around 5 minutes and providing an increase in productivity of 30% resulting from the improved control.

It is worth noting that improved GC analysis times via method development has reduced the original analysis time from 35 minutes to around 10 minutes, making this development less important, given the expense of the equipment involved.

1.2.2.3 Product inhibition

Product inhibition of (1S,5R)-2-oxa-bicyclo[3,3,0]oct-6-en-3-one (reaction scheme given in Figure 1.5) occurred at around 3 g/L (Doig *et al.*, 2003).

The substrate and product are not only inhibitory to the CHMO enzyme, but are also toxic to the *E. coli* cells at the higher concentrations at which bioconversion is inhibited. This prevents reuse of the biocatalyst and makes it an expensive stoichiometric reagent.

To lessen the impact of product inhibition, two approaches of *in-situ* substrate supply and product removal (ISSSPR) have been attempted by Simpson *et al.* (2001) and Hilker et al. (2004a and b):

- Use of a biphasic mixture where the product is extracted into the organic phase to prevent the product concentration exceeding inhibitory concentrations - various

organic solvents were tested (*n*-octane, cyclohexane, isopropyl ether, hexane, dichloromethane, *n-tert*-butyl methyl ether, ethyl acetate, toluene), but only *n*-octane marginally improved the biotransformation rate and yield (to 4.5g/L) with 40% of the lactone being extracted from the aqueous layer. The other solvents were both ineffective and harmful to the cells.

- Use of an adsorbent resin, charged with the substrate prior to being used in a reaction. Equilibrium between the resin and the aqueous reaction medium both supplies the substrate and removes the product via an external loop (Simpson, 2001) or by being directly charged into the reaction medium (Hilker et al, 2004a and b), preventing the concentration of either from becoming inhibitory. Various resins were tested and a maximum product concentration of 21g/L was achieved when using two batches of a triple strength biomass concentration in an oxygen bubble reactor.

1.2.2.4 Cofactor recycling

The enzyme requirement for cofactors has already been explained in section 1.1.6. Taschner *et al.* (1993) studied isolated CHMO by supplying stoichiometric amounts of NAD(P)H. This method is too expensive to employ at scale.

The use of a secondary enzyme is one method of overcoming this as described in section 1.1.6. Rissom *et al.* (1997) used a secondary enzyme, formate dehydrogenase, to regenerate NAD(P)H with CHMO isolated from the wild type host (*A. calcoaceticus*). Three batch-wise recycles were performed with relatively constant reaction rates and high *ee* (>99%). Zambianchi *et al.* (2002) isolated CHMO from *Thermoanerobium brockii,* immobilised it on Eupergit C, and used the secondary enzyme system alcohol dehydrogenase and 2-propanol to enable sixteen, 24 hour recycles with 5g/L of substrate thioanisole converted on each recycle.

However in this latter successful result, it is noteworthy that alcohol dehydrogenase is still relatively expensive, is more difficult to employ than whole cell biocatalysis and

thus it has been often stated that whole cell biocatalysis is preferable for use at scale (Doig *et al.*, 2003, Simpson *et al.*, 2001, Hilker *et al.*, 2004a & b).

1.2.2.5 Intracellular enzyme stability

The main impact seen on the enzyme stability has been the inhibitory nature of the substrate and product, as described above. The maximum time for which enzyme activity was maintained during a bioconversion with whole cells was assessed to be 10 hours with the ISSFPR of Hilker *et al.* (2004b).

A recent investigation into whether the lack of enzyme stability was caused by the cloning of CHMO into *E. coli* TOP10 [pQR239] or is inherent in the wild type host (*A. calcoaceticus*) showed that the CHMO isolated from both sources was practically identical (Secundo *et al.*, 2005). This indicates that the lack of enzyme stability observed in *E. coli* TOP10 is not due to any genetic modification of the enzyme or change in host.

Enzyme stability can be increased by either using an alternative cell host as described in section 1.2.2.1 or by immobilisation as described in section 1.1.6. The first attempt to employ directed evolution in the generation of a new Baeyer-Villiger monooxygenase has recently been published by Bocola *et al.* (2005) who took phenylacetone monooxygenase (PAMO), a relatively stable BVMO which accepts only a narrow range of substrates and produces relatively impure products, and increased both the range of substrates accepted and the enantiopurity of the product whilst maintaining the enzyme stability.

1.2.2.6 Further factors limiting industrial application

Volumetric productivity and thus economic feasibility of the biocatalytic reaction remains low, with a maximum product concentrations of 16g/L obtained by Simpson *et al.* (2001) and 21g/L obtained by Hilker *et al.* (2004b) with cell concentrations of 10g/L and approximately 30 g/L respectively. Whilst it is not simple to quantify, there

is an obvious trade off between using high biomass concentrations (with high fermentation costs) and lower biomass concentrations and the increased downstream processing costs that result.

1.3 Scale-up of drug metabolite production

1.3.1 Drug development and ADMET

The average drug is estimated to have a Research and Development cost of $800m (Rawlins, 2004) with a third of this cost relating to clinical trials. Of the drugs that reach clinical trials, over 90% still fail. The R&D spend of most large pharmaceutical companies runs into billions of dollars per year (ArQule spent $2.1b in 2002) and the need for improved efficiency is clear.

Before a bioactive molecule can become a drug it needs to simultaneously possess favourable absorption, distribution, metabolism, excretion and toxicity (ADMET) properties. The iterative process of screening for such properties is shown in Figure 1.7.

It is widely recognised that the study of a drug's ADMET properties helps to prevent their failure before they reach clinical trials (Ekins, 2005; Lipinski, 2000). In 1997, ADMET 'liabilities' accounted for 39% (Figure 1.8) of clinical trial failures. For this reason many pharmaceutical companies (e.g. ArQule) are strategically bringing drug development processes in parallel with early ADMET screening to give rise to better potential drug candidates whilst providing a fast fail route of many other potential leads. (A review of ADMET technology is given at http://www.netsci.org/Science/Special/feature06.html).

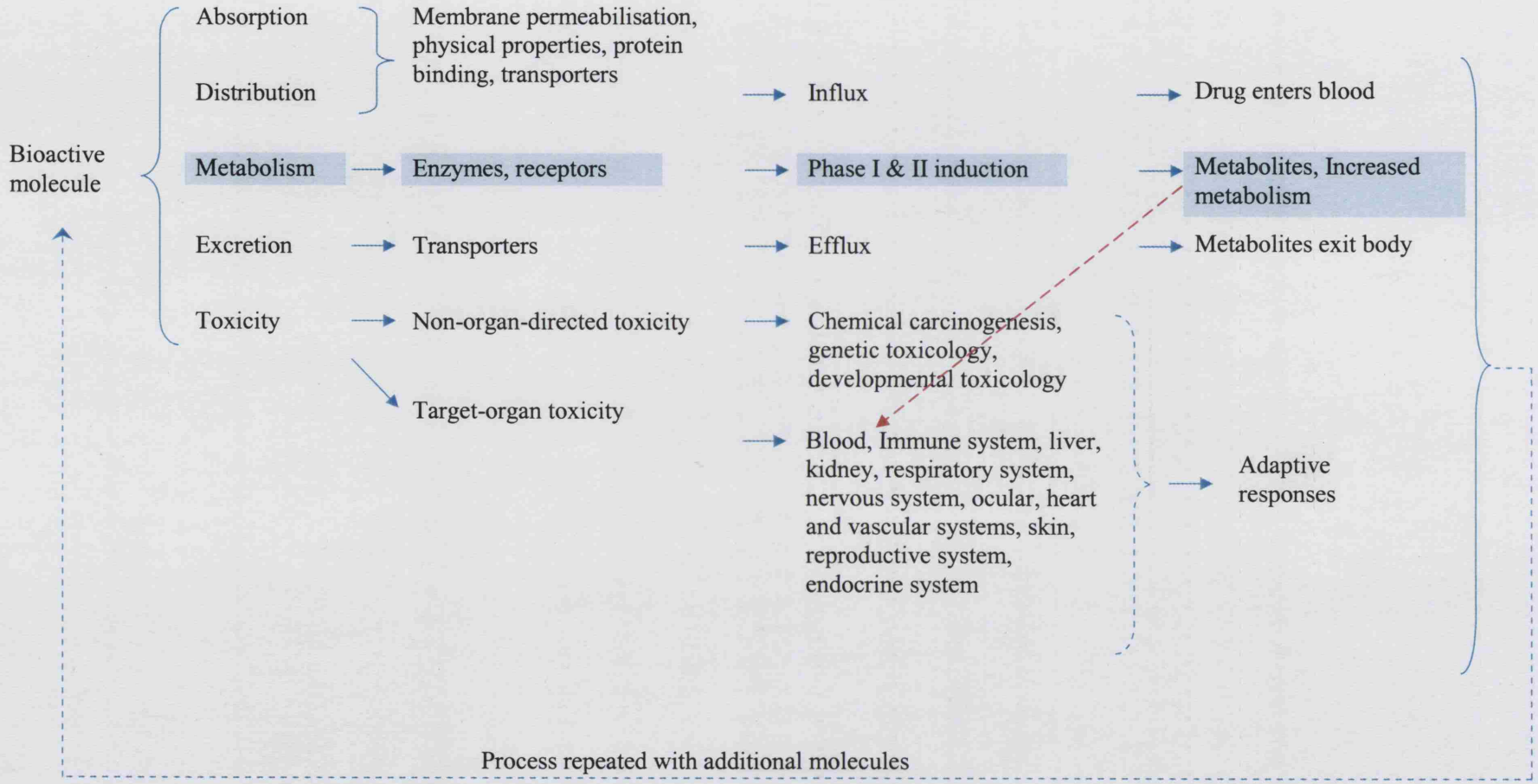


Figure 1.7: The iterative ADMET optimisation process. Adapted from Ekins *et al.* (2005). The red dotted arrow represents reactive metabolites that can cause toxicity.

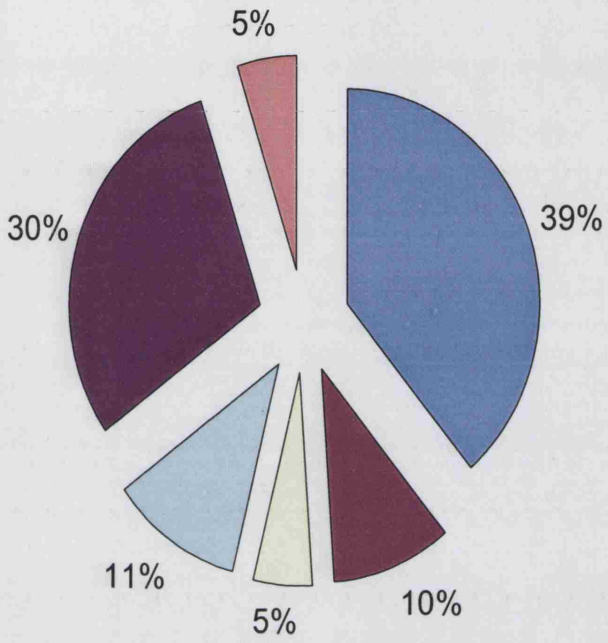


Figure 1.8: Reasons for the clinical failure of drug candidates as a proportion of 198 new chemical entities. Adapted from Kennedy (1997).

ADMET, Adverse effects in man, Commercial reasons,

Animal toxicity, Lack of efficacy, Miscellaneous.

In the human body, metabolism is responsible for the time for which an administered drug is active and as such is critical to its efficacy. Metabolites are produced with higher aqueous solubility, allowing a greater amount of the foreign substance to be excreted in urine or bile (Tredger and Stoll, 2002). Metabolism generally occurs in two stages (Zimmerman, 1999):

- Phase I metabolism: Small polar groups are introduced to the parent drug which increases the aqueous solubility.
- Phase II metabolism: Conjugates are formed by adding large polar compounds to these polar group

Phase I metabolism can involve oxidation, reduction or hydrolysis. The oxidative metabolism is predominantly attributable to cytochrome P450s (CYPs).

There are four potential types of drug metabolites, these and their effect are shown in Figure 1.9. Active metabolites are worthy of particular attention as they can enhance, modify or inhibit the desirable activity of a drug. Sometimes the metabolite itself has the pharmacological activity (pro drugs), which is useful when the active metabolite itself is poorly soluble or unstable. For example, the antihistamine Terfenedine was withdrawn from use due to its fatal interaction with erythromycin and ketoconazole. Terfenadine's active metabolite fexofenadine, which does not cause fatal drug-interactions, is now used instead (Pratt *et al.*, 1999). In contrast toxic metabolites are often a cause of many drug-candidates being dropped in the early stages of the drug discovery process.

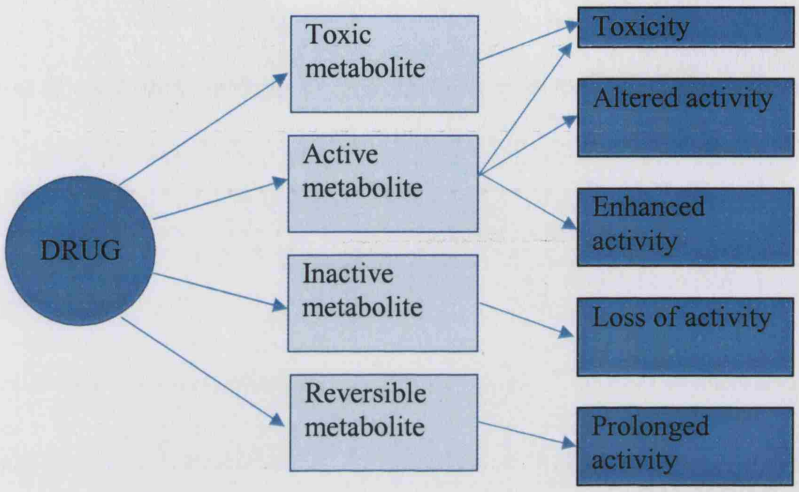


Figure 1.9: The end results of drug metabolism. Adapted from Gunaratna (2000a).

1.3.2 Metabolite Screening

Currently Phase I CYP metabolism is regarded as the most important in drug development, with four aspects generally studied (Fiehn, 2001; Crespi and Miller, 1999):

1. The overall rate of metabolism. This metabolism influences pharmacokinetic properties, such as bioavailability and clearance, in both humans and preclinical animal species. This is generally studied in vitro using isolated hepatocytes, liver slices, or tissue fractions (e.g. microsomes fortified with the necessary cofactors); these are further described in sections 1.33 and 1.34.
2. The metabolite profile. This is important for selection of an appropriate species for preclinical safety trials (i.e. are the metabolites formed in humans also formed?).
3. Identification of the CYP enzyme(s) involved in metabolism. This is important to enable establishment of the expected variability in metabolism in different patients, with high degrees of variability requiring careful patient monitoring. It also allows prediction of drug-drug interactions. Generally if more than one enzyme metabolises a drug there will be less variability and a reduced chance of drug-drug interaction as inhibition of one CYP enzyme will not prevent metabolism by others.
4. The CYP enzyme inhibition by the drug. For example, a drug may inhibit a CYP that is not significantly involved in its metabolism, further increasing the risk of drug interactions.

To assist with the study of metabolism and the key issues of CYP induction, inhibition and drug-drug interactions, several commercial databases have been created using published data which are useful for predicting drug metabolism:

- Metabolite™ (http://www.mdl.com)
- Metabolism™ (http://www.accelrys.com)
- BioFrontier/P450™ (http://www.fqspl.com.pl/)
- MetabolExpert™ (http://www.compudrug.com/)
- META™ (http://www.multicase.com/)

- METEOR™ (http://www.chem.leeds.ac.uk/luk/)

The empirical data from these databases have been used to build computational models and 'score' many virtual molecules for enzyme inhibition (Etkins, 2003).

1.3.3 Cytochrome P450s

Cytochome P450s are a large group of membrane bound, heme-containing mixed-function oxygenases responsible for the primary metabolism of toxic hydrocarbons (Crespi and Miller, 1999). These enzymes are expressed in many tissues, the predominant source being the liver. The principle function of CYPs is to introduce oxygen into a molecule to increase the hydrophilicity of the product and thus the ease with which the product can be eliminated from the body.

The CYPs activity requires molecular oxygen and the cofactor NAD(P)H. Microsomal CYPs require electron transfer from NAD(P)H via the flavoprotein NAD(P)H:CYP oxidoreductase (OR). Additionally, cytochrome b_5 (a membrane-bound protein similar to CYPs) can stimulate the CYP activity for some CYPs and their substrates (Thummel and Wilkinson, 1998). Therefore, the catalytic activity of a CYP is determined not only by the amount expressed, but also by the abundance of its cofactor.

In humans there are around twelve families (CYPs 1-12) and seventeen sub-families (designated by a letter) of CYP enzymes, though only four families (CYPs 1-4) are responsible for the majority of drug oxidations (Kruijtzer *et al.*, 2002). Sakaki and Inouye (2000) reviewed the practical potential applications of human cytochrome P450s.

CYPs can be induced by drugs and inhibited by them. Many drug-drug interactions also occur, which can lead to the time-dependent over- or under-metabolism of the drugs. For example, alcohol acutely inhibits the CYP2E1 metabolism of paracetamol but is also a long-term inducer of the same enzyme (Tredger and Stoll, 2002).

1.3.3.1 CYP3A4

CYP3A4 is the most important human enzyme in the first-pass oxidative metabolism of drugs due to its high expression level in the liver and its broad substrate specificity (Ekins *et al.*, 2003). CYP3A4 is the most abundant CYP450 enzyme in the human liver, representing 30% of the total CYP content by weight (Plant and Gibson, 2003), and is significantly expressed in the small intenstine. Not surprisingly, given its relative abundance, CYP3A4 is responsible for the metabolism of around 60% of drugs in man (Lamba *et al.*, 2002; Zhou *et al.*, 2005). (For a detailed review of CYP3A enzymes and drug interactions see Thummel and Wilkinson, 1998).

Most CYP3A4 substrates do not obey classical Michaelis-Menten kinetics and three different mechanisms of inhibition have been shown (see section 1.4.1). As such, *in vitro* prediction of drug clearance and drug-drug interactions (including inhibition) are often very difficult and inaccurate. The crystal structure of CYP3A4 has only recently been reported (Williams *et al.*, 2004), but this should assist in understanding the complexity of CYP3A4 inhibition through quantitative structure-activity relationships (Ekins *et al.*, 2003). A multisite kinetic model has been proposed for the active site (Galetin *et al.*, 2002) and the atypical kinetics modelled (Houston and Galetin, 2005).

There are numerous different drugs metabolised by CYP3A4 and standard probe substrates are commonly used, the most common being testosterone (Yuan *et al.*, 2002). In this study, verapamil (Figure 3.3), an alternative and commonly used substrate, was employed as a test probe.

1.3.4 In vitro CYP sources

A comparison of the different *in vitro* systems that can be used in the study of drug metabolism is given in Table 1.8. Each of the systems is described in more detail below.

System	Advantages	Disadvantages	Future Needs
Hepatocytes	Integrated cellular system	Short life time, limited enzyme stability	Increased availability, improved cryopreservation
Liver slices	Easy to prepare, cellular integrity maintained	Limited medium penetration, short-term viability	Increased availability, improved cryopreservation
Microsomes	Well-used, long-term storage possible at -80°C, well characterised	Limited information, cofactor requirement	
Expressed enzymes	Pure system	Single enzyme	Integration with other enzyme systems
Micro-organisms	Rapidly grown single enzyme systems	Limited information, cofactor requirement	

Table 1.8 Comparison of CYP expressing *in vitro* systems. Adapted from (Gunaratna, 2000a).

1.3.4.1 Hepatocytes and liver slices

The most complete way of studying hepatic metabolism can be obtained by using intact liver systems, in which cofactor recycling is self-sufficient and the natural orientation of linked enzymes is preserved. The main advantage of such systems is that Phase II metabolism can be studied alongside Phase I metabolism, and therefore the results are often more comparable with *in vivo* results than those using liver microsomes and are the preferred *in vitro* system (Gunaratna, 2000a).

The two usual intact fresh liver systems studied are isolated hepatocytes and liver slices. However the major negative factor of these systems is the longevity of their enzyme activity, which is not stable for much more than a day. With the increasing availability of fresh human tissues, human hepatocytes have become the most complete solution and a viable alternative to studying metabolism in liver microsomes. However, freshly isolated hepatocytes can suffer from over a twofold batch-to-batch variation, which makes them unsuitable for use in high-throughput assays in drug development (Li, 2004).

1.3.4.2 Human Liver Microsomes

Microsomes are a subcellular fraction of tissue (usually the liver) prepared easily by differential high-speed centrifuge extraction. Liver microsomes provide the most convenient and most widely used way to study CYPs as they contain all of the CYP enzymes and maintain their activity for several years when stored at -80°C. As with most isolated oxidative enzymes, CYPs require a cofactor such as NAD(P)H.

The concentrations of CYP450 enzymes in microsomes can vary between people and particularly between ethnic groups. It is therefore best to use a pooled source of microsomes to ensure that all CYP450 enzymes are present, unless having a deficiency in one or more metabolic pathways is the object of the study. Alternatively to manipulate the CYP levels expressed selective chemical inducers and inhibitors can be used (Gunaratna, 2000a).

1.3.4.3 Recombinant CYP enzymes

CYPs and CYP3A4 in particular have been expressed in a number of cell systems (Table 1.8). Like other oxidative enzymes it requires the cofactor NAD(P)H, therefore the most efficient recombinant systems usually coexpress CYP3A4 with NAD(P)H-P450 reductase. The coexpression of human cytochrome b5 has also been shown to enhance CYP3A4 activity, with baculovirus-transfected insect cells (Gonzales *et al.*, 1991) and *E. coli* (Hagen *et al.*, 2002; Yamazaki *et al.*, 2002) having been used for such co-expression. (See Gonzalez and Korzekwa (1995) for a review of the different hosts used for CYP expression).

Bacterial or yeast expression of CYPs has the advantage that system development is rapid and that the culture medium is inexpensive relative to mammalian or insect cell systems. Such systems are thus ideal for the isolation of large quantities of enzyme or for use in the production of large quantities of metabolites (Yamazaki *et al.*, 2002). Baculovirus expression appears to offer a higher CYP content, with a high catalytic activity per unit CYP enzyme. However, because the enzymes are produced transiently in the host cells, the harvest time and other culture conditions can have a significant effect on the CYP activity (Gonzales *et al.*, 1991). Mammalian cell lines (HepG2, CHO and Human lymphoblastoid cells) have also been relatively successful, though most CYP enzymes appear to adversely affect the growth of the host cell and impose an upper limit on the expression level. Mammalian cell expression offers the possibility to couple CYP metabolite formation to toxicological endpoints (i.e., cytotoxicity, malignant transformation, or mutagenesis investigations) (Andrews *et al.*, 2002). Andrews *et al.* (2002) compared the expression of CYP3A4 in *S. cerevisiae*, *E. coli* and human lymphoblastoid cells as alternative systems to human liver microsomes and found that the human lymphoblastoid cells gave reaction rates three to nine fold higher than the other systems (including microsomes).

An interesting result was recently published by Ahn and Yun (2004) who co-expressed CYP3A4 with HDJ-1, a molecular chaperone in man known to assist the correct folding of unfolded proteins. This gave over a three-fold increase in CYP3A4 expression and a fifteen fold increase in its catalytic activity.

Recombinant cell	Reference
S. cerevisiae	Andrews *et al.*, 2002; He and Chen, 2005, Murray and Correia, 2001
E. coli	Andrews *et al.*, 2002; Ahn and Yun, 2004; Hagen *et al.*, 2002; Yamazaki *et al.*, 2002
Baculovirus-transfected SF9 cells	Gamble *et al.*, 2003; Gonzales et al., 1991
HepG2 cells	Omasa *et al.*, 2005; Araki *et al.*, 2003
Chinese hamster ovary (CHO)	Ding *et al.*, 1997
Human lymphoblastoid cells	Andrews *et al.*, 2002; Gamble *et al.*, 2003

Table 1.9: Published CYP3A4 expressing recombinant cell systems.

1.3.5 Enzyme kinetics

Enyzme kinetics are generally expressed in terms of the Michaelis-Menten equation (Voet and Voet, 1995).

$$\text{Rate of reaction (v)} = \frac{V_{max}[S]}{K_m + [S]}$$

Sonnad and Goudar (2004) describe the several ways to solve this equation for determination of V_{max} and K_m from experimental data and their inherent limitations:

- The linearization by algebraic manipulation of the equation and graphical representation which is most commonly applied their parameter estimates are often highly inaccurate due to the inherently non-linear nature of the equation.
- Progress curve analysis (i.e. plots of substrate or product concentration over time) overcomes this problem (Duggleby, 1995), but can involve computational difficulties.
- An explicit solution of the Michaelis-Menten equation has been derived by computer algebra (Schnell and Mendoza, 1997), though complex mathematical models are required which are outside the scope of this study.

The well established method of taking the initial slope of the progress curve (Cornish-Bowden, 1995) is the method adopted throughout this study. Whilst this yields reliable evidence of initial reaction rates, it is noted that much progress curve data is unused by the implementation of this method. Yeow *et al.* (2004) detail a complex mathematical approach to utilising the entire progress curve to yield potentially more meaningful reaction rates. Furthermore it is noted that when the Michaelis-Menten equation is applied to whole cell oxygenase bioconversions, with significant levels of inhibition, atypical kinetics features are often seen.

1.3.6 Inhibition

Inhibition results from molecules binding to enzymes and results in a decrease in their activity, it is usually reversible (Xenotech, http://www.xenotechllc.com/). The decrease in enzyme activity can be as a result of substrate or product concentration, the extent being specific to the substrate or product. Inhibition is particularly common and thus important in oxygenase enzyme biocatalysis.

1.3.6.1 Types of enzyme inhibition

Three types of inhibition are observed in catalysis:

- Competitive Inhibition - Where the inhibitor directly competes with the substrate for the active site of the enzyme. Due to the structural similarity of a substrate and its product, product inhibition is often competitive. This type of inhibition can potentially be overcome by sufficiently high concentrations of the substrate.
- Non-competitive inhibition – where the inhibitor binds to the enzyme at a location away from the active site, but in doing so structurally distorts the enzyme and thereby renders the enzyme catalytically inactive or reduces the reaction rate. Therefore the extent of inhibition depends only on the inhibitor concentration.
- Uncompetitive (or mixed) inhibition – where the inhibitor binds to a site that only becomes available after the substrate has bound to the active site. This

inhibition is most commonly seen with multi-substrate reactions, with a product frequently being the inhibitor. High levels of substrate concentrations can also cause uncompetitive inhibition, when multiple substrate molecules try to bind to an active site meant for only one. This type of inhibition can be reduced, but not overcome, by increasing the substrate concentration.

This model has also been applied to Baeyer-Villiger biocatalysis by Moonen *et al.* (2005), the derivation of the various constants requiring non-linear regression analysis (Sonnad and Goudar, 2004; Moonen *et al.* 2005). In this study comparisons between k'_{obs} at different substrate concentrations will be made for CHMO, which will allow comparison of the inhibition seen. It is the qualitative effect of CYP inhibition that is important for the study of metabolite scale-up potential, and quantitative analysis is not studied.

CYP inhibition can be categorised further and can involve (Tredger and Stoll, 2002):

1. Simple reversible competitive inhibition between two substrates at the CYP active site. Ketoconazole, an antifungal agent and well-known inhibitor of CYP3A4 displays such short-lived inhibition and was selected as the inhibitor of CYP3A4 activity used in chapter 3.
2. Competition between one drug and the metabolite of the other produced at the active site.
3. Irreversible effects of a reactive metabolite of a drug on the CYP producing it, thus preventing the metabolism of other drugs (also known as mechanism-based or suicide inhibition). Mechanism based inhibition is responsible for many drug-drug interactions. Verapamil is known to demonstrate such inhibition of CYP3A4 (Zhou *et al.*, 2005) and is used as a probe substrate in chapter 3.

The inhibition of CYP-mediated metabolism is often the mechanism of drug-drug interactions (Crespi *et al.*, 1997). This has led to a great deal of study of its kinetics and inhibition. CYP3A4 often demonstrates positive cooperativity and/or activation, and conflicting results are often seen for different substrates (Galetin *et al.*, 2002; Stresser *et al.*, 2000; Wang *et al.*, 2000). Galetin *et al.* (2002) suggested that the different kinetics seen with alternate substrates is attributable to multiple binding sites.

Houston and Galetin (2005) describe the two alternative options to quantifying CYP3A4 kinetics to be an empirical approach and a mechanistic approach, the more complex latter approach being the more useful. Kenworthy *et al.* (2001) explain that the interactions between substrates and inhibitors of CYP3A4 are complex and difficult to predict given the current understanding of this enzyme. As with other complex computational kinetic modelling, the results of which have still to gain common acceptance, such studies are outside the scope of this study.

Michaelis-Menten kinetic equations have been derived for the various types of inhibition (Cornish-Bowden, 1995). However more complex models are required for the atypical kinetics apparent with substrate inhibition. Such models require computation data fitting and parameter estimation (Tracy and Hummel, 2004) and as such, though they will be briefly explained, have not formed part of this study.

When substrate inhibition is seen, the following variant of the Michaelis-Menten equation, the Haldane model is generally used (Chung *et al.*, 2003):

$$k_{obs} = \frac{k_{cat}[S]}{K_m + [S] + ([S]^2 / K_s)}$$

Where k_{obs} is the apparent initial rate observed, k_{cat} is the apparent maximal rate at saturating substrate concentration, K_m is the apparent Michaelis constant for the substrate and K_s is the apparent secondary-binding inhibition constant for the substrate. A higher K_s value implies that the culture is less sensitive to substrate inhibition.

1.4 Aims of the Project

An EngD differs from a PhD in that it requires that projects include taught elements, include research both at university and the sponsoring company and also include a business component.

The development of pharmaceuticals requires expertise from a diverse range of scientific areas. Some areas have been identified where this process can potentially be enhanced:

- The opportunity for improved drug purity, as offered by biological synthesis routes, is often missed due to perceived problems with inhibition and the integration of biocatalysis with chemical reaction steps.
- The failure rate of drug candidates in clinical trials remains high. Increased early (pre-clinical) use of ADMET testing could be employed to fast fail potential drug candidates early if sufficient quantities of metabolites could be made available.
- Other than drug portfolio diversification, little or no strategy is often involved in the management of financial risk, with many key business decisions being made based on intuition or trial and error.

To address these perceived areas of weakness, four different pharmaceutical development processes were studied in this project:

1. Process development – Characterisation of the effect of inhibition in oxygenase biocatalysis and the effectiveness with which immobilised whole cell biocatalysis can meet the need for simpler integration with chemical reaction steps. These studied are described in Chapter 2.
2. Pharmaceuticals testing - Drug metabolite identification and metabolite production. The model system used, as discussed in chapter 3, was the CYP3A4 catalysed metabolism of verapamil.
3. Business strategy – an analysis of business decision making in the pharmaceuticals development process, based upon the strategic development of a start up contract research organisation. These studies are described in Chapter 4.

4. Process validation – the increasing role of regulation and the importance of validation in the pharmaceutical development will be addressed with reference to the above three studies. These studies are described in Chapter 5.

2 The potential problem of CHMO inhibition

2.1 Aims of the chapter

- To compare the two available *E. coli* cells hosts, TOP10 (pQR239) and JM107.
- To determine the effect of immobilisation of whole cells on enzyme stability
- To assess the scale-up potential of the immobilisation process.
- To compare the inhibition by different substrates in free cells, immobilised whole cells and isolated enzyme bioconversions.
- To assess the suitability of flow cytometry for cell viability and enzyme inhibition determination for whole cell oxygenase biocatalysts.

2.2 Materials & Methods

2.2.1 Reagents and suppliers

Reverse osmosis (RO) water was used in all experimental work. All other reagents used were of analytical grade and were obtained from Sigma (Poole, Dorset, UK), apart from the yeast extract (Oxoid, Basingstoke, Hants., UK) and bis-(1,3-dibutylbarbituric acid) trimethine oxonol (BOX) (Molecular Probes, Eugene, OR, USA). The Bicyclo[3.2.0]hept-2-en-6-one was a generous gift from Sigma (Poole, Dorset, UK).

2.2.2 Biocatalyst production

2.2.2.1 Storage and maintenance

E. coli TOP10 pQR239 and *E. coli* JM107 were gifts from Dr John Ward, Department of Biochemistry and Molecular Biology, University College London. Master stock cultures of the two *E. coli* strains were stored at -80°C on agar plates containing LB nutrient broth and 100 mg/L ampicillin. New working stock culture plates were

prepared from a single colony from the master stock plates monthly. This colony was aseptically transferred into a sterile 1 L shake flask containing 100 ml of the growth medium described in Table 2.1, to which 100 mg/L ampicillin had been added via a 0.2 μm filter (Pall, Portsmouth, Hants., UK). When the culture had grown to approximately 1 g/L, 50 % v/v of sterile glycerol was aseptically mixed and 1 ml aliquots were grown on individual plates. These working stock cultures were used throughout this work.

2.2.2.2 Inoculum growth

2L baffled shake flasks containing 500 ml of the medium described in Table 2.1 were autoclaved at 121 °C for 20 minutes. When the flask had cooled, it was adjusted to pH 7 and ampicillin was added via a 0.2 μm filter (Pall, Portsmouth, Hants., UK) to a concentration of 100 mg/L. An individual cell colony was aseptically transferred from the working stock culture plates described in Section 2.2.1.1. and incubated overnight at 37 °C in a Model G25 orbital shaker at 200 rpm (New Brunswick Scientific, Edison, New Jersey, USA).

Media component	Concentration (g/L)
Yeast extract	25.0
Peptone	10.0
Glycerol	12.0
NaCl	10.0

Table 2.1: Composition of the growth medium used in all *E. coli* TOP10 [pQR239] and *E. coli* JM107 inoculum flasks and fermentations.

2.2.2.3 E. coli Fermentation

Fermentations were routinely carried out in a LH 7L stirred tank fermenter with a 5L working volume (Inceltech/LH Fermentation Ltd., Reading, Berks., UK). The fermenter itself comprised a glass vessel with an aspect ratio of 1.63, two six-bladed

Rushton turbine impellers and four equally spaced baffles. The ratio of the vessel diameter to the impeller diameter was 3.2. The pH was measured by a steam sterilisable Ingold pH probe (Ingold Messtechnik, Urdorf, Switzerland) and was maintained at pH 7 (± 0.05) by the metered addition of 4M NaOH and 4M H_3PO_4. Dissolved oxygen tension (DOT) was measured by a polarographic oxygen electrode (Ingold Messtechnik, Urdorf, Switzerland). The vessel was aerated with 0.67 vvm sterile air via a submerged sparger, and the temperature was maintained at 37 °C using a temperature probe and heating element (Bioprocess Engineering Services Ltd, Sittingbourne, Kent, UK). Antifoam (polypropylene glycol 2000, 4 mL) was added prior to the start of the fermentation to give a final concentration in the fermenter of 0.8 mL/L.

The composition of the complex growth medium used was the same as for inoculum preparation and is shown in Table 2.1. The fermenter, initially containing 4.5L of medium, and ancillary equipment was steam sterilised *in situ* at 121 °C for 20 minutes. After cooling the fermenter to 37 °C, and just prior to inoculation, an aqueous ampicillin solution was added to the fermenter by filtration through a 0.2 μm filter to a final concentration of 50 mg/L. The fermenter was then inoculated with 500 ml of overnight culture (10% v/v inoculum), prepared as described in Section 2.2.1.2. The progress of the fermentation was followed by taking OD_{670} measurements, as described in Section 2.2.4.1. When the OD_{670} measurement reached 11 to 12, for the *E. coli* TOP10 pQR239 cells, concentrated L-arabinose solution was added via a 0.2 μ m filter to a final concentration of 0.2% w/v to induce the expression of CHMO. For the *E. coli* JM107 cells, IPTG was added to a final concentration of 1mM to induce the CHMO expression.

Initially the impeller speed in the fermenter was set at 800 rpm in order to maintain the DOT above zero percent, until the cell density reached around an OD_{670} of 7 to 8 and DOT reached zero. At this DOT the impeller speed was increased to 1000 rpm. When the DOT percentage again reached zero, oxygen became growth limiting, however an excess of oxygen during enzyme expression has been previously shown to be damaging to the CHMO activity (Doig *et al*, 2001a). Three hours after the CHMO

enzyme was induced, the fermentation was complete and the cells were harvested from the fermenter.

The inlet and exhaust gases were filtered through 0.2 μm filters (Gelman Sciences, Ann Arbor, MI, USA) and the composition of the exhaust gas was determined by a mass spectrometer (Prima 600, VG Gas Analysis, Middlewich, Cheshire, UK) controlled by a VG gas analysis microprocessor (MM8-80F, VG Gas Analysis, Middlewich, Cheshire, UK). Data logged from the fermenter itself, e.g. impeller speed, pH, DOT etc. and exhaust gas measurements were recorded with the RT-DAS program every minute (real-time data acquisition system) (Acquisition Systems, Guildford, Surrey, UK). A typical fermentation profile is given in Appendix I.

The biomass resulting from the fermentation was either used in the same medium to carry out a resting cell bioconversion (section 2.2.5.2), employed in the isolated enzyme reaction (section 2.2.3.1), or if a wet cell paste was required for whole cell immobilisation (section 2.2.3.2), the cells were harvested by centrifugation at 5000 rpm for 20 minutes at 4 °C in 2.5L batches (Heraeus Megafuge, Heraeus Instruments Ltd, Brentwood, Essex, UK).

2.2.2.4 Biocatalyst storage

Subsequent to the fermentation (section 2.2.1.3), cells were stored in the fridge for up to 4 days prior to use in subsequent biocatalysis experiments. This was in line with work performed by Walton and Stewart (2002), which showed that for up to a week after fermentation the CHMO activity of the cells remained constant.

2.2.3 Biocatalyst preparation

2.2.3.1 Enzyme isolation

A 1 ml sample of fermentation broth in a 1.5 mL Eppendorf tube was centrifuged at 13000 rpm for 2 minutes, the supernatant was discarded and the cell pellet resuspended in 1 mL of 50 mM sodium phosphate buffer, pH 7. The cells were disrupted by sonication at an amplitude of 8 μm using a Soniprep 150 MSE (Sanyo, Crawley, W. Sussex, UK) with a cycling programme of five, 10 second on/off bursts. During the sonication procedure the sample was placed in an ice bath to prevent it from heating up. The sonicated sample was finally centrifuged at 13000 rpm for 2 min to remove cell debris and the resulting crude CHMO solution was kept on ice prior to use.

2.2.3.2 Whole cell immobilization

Statistically designed experiments

Statistical design of experiments and subsequent analysis of results was performed using Design Expert 5 (Stat Ease, Inc., Minneapolis, USA).

Cell paste was extracted from the *E. coli* TOP10 fermentation broth by lab scale centrifugation (15 minutes, 5000 rpm, 4 °C, (Heraeus Megafuge, Heraeus Instruments Ltd, Brentwood, Essex, UK)). An 8% w/v solution of sodium alginate was prepared and mixed overnight. This was used to make 2, 3 and 4% w/v sodium alginate encompassing different amounts of cell paste (measured on a dry cell weight basis) to give cell concentrations of 0.01, 0.07 and 0.13 g(DCW) cells/g bead. Water was then used to maintain equal volumes of sodium alginate-cell pastes between experiments and the final sodium alginate concentrations quoted. The resulting alginate-cell pastes were then dropped from 50mL syringe housings fitting with pipette tips from 40cm height (experimentally determined to be the optimum height for spherical bead formation) into either 0.05, 0.5 and 0.95M of stirred calcium chloride. The resulting beads were either recovered immediately using a filtration cloth or left in the fridge to

harden for 15 or 30 minutes prior to recovery. Recovered beads were washed in water and resuspended in complex media.

Subsequent studies

In experiments subsequent to the statistical experimental design, the bead size was controlled by a parallel gas flow during the pumped dropping of alginate-cell paste into calcium chloride (see Figure 2.1). Bead size was manually measured as the average width of fifty beads. From triplicate measurements the coefficient of variance of this manual technique was found to be ±5%.

2.2.4 Cell separation methods

For the large-scale separation of cells used in the scale up of whole cell immobilised bead production, two techniques were employed; cross flow filtration and centrifugation.

For cell recycle studies, the lab scale separation of cells was performed by bench top centrifugation and for the recycling of immobilised whole cell beads a filtration membrane was used.

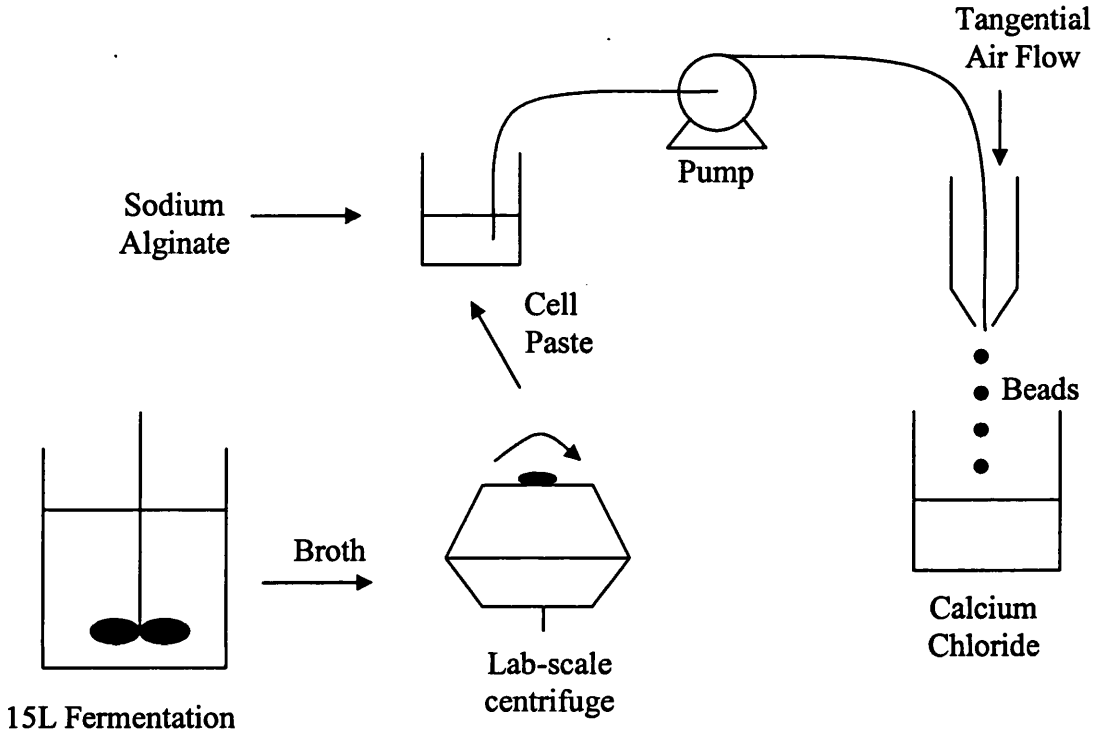


Figure 2.1: Overview of the whole cell immobilisation process. The tangential flow of air disrupts bead formation, forcing them to drop out of the tubing before they reach the larger sizes required to fall by gravity alone.

2.2.4.1 Crossflow filtration

Crossflow filtration was performed in a ProFlux™ M12 rig (Millipore Corporation, Bedford, MA, USA). Two Masterflex™ L/STM (Cole-Parmer, Instrument Company, Vernon Hills, IL, USA) peristaltic pumps were incorporated; the retentate circulation pump was an Easy-Load™ II Model 777201-62 and the permeate flux control pump was a Quick-Load™ Model 70201-24. The process tank was kept at 10°C using a cooling jacket. The rig also included three pressure transducers to measure inlet, outlet and permeate pressures connected to a digital display. A schematic is shown in AppendixII, Figure AII.1.

The filtration module used was a 0.1 m^2 cassette (Durapore, Pellicon 2 Mini) fitted into a stainless steel cassette holder (Millipore Ltd, Watford, Herts., UK). The membrane had nominal pore size of 0.65 μm and open channels with turbulence screens (v-screen) designed to minimise foulant deposition.

The recirculation and permeate pumps were gradually ramped up to give a crossflow rate of 0.4 m/s and permeate flux of 25 L/m^2h respectively. Constant transmembrane pressure (TMP) was achieved by adjusting the permeate pump speed. The inlet, outlet and permeate pressures were also monitored throughout the process, as was the permeate flux.

After 60 minutes of operation at approximately 15 psig, a 1.5-fold concentration was achieved. This concentration is sufficient to produce the calcium alginate beads by use of 8 % w/v sodium alginate. The pseudo steady state flux was a very low value of 3 L/m^2h. However, it should be noted that no attempt was made to optimise the filtration operating conditions, nor membrane type. Filtration performance is detailed in Appendix II, Figure AII.2

Post-filtration, the membrane system was cleaned with 0.1M phosphoric acid, at 45°C, for 20 minutes. Pure water flux was measured at different operating conditions, after cleaning, to assess the effectiveness of the cleaning step. The resulting pure water

flux was fully recovered after cleaning, indicating that the membrane was not severely fouled, and multiple re-use would be possible.

2.2.4.2 Pilot plant scale centrifugation

The pilot scale SC-6 disk stack centrifuge installed with a hydro-hermetic 'soft' feed zone was used for the duration of this study. An overhead feed pressure of 0.4 bar (gauge) was maintained when feeding cell suspension into the centrifuge, a requirement for flooding the feed zone and activating the hydro hermetic feature. The operating water for controlling the discharge mechanism was supplied and maintained at a pressure of 4.5 bar via a Grundfos CR 2-50 pump (Grundfos pumps Ltd., Leighton Buzzard, Beds., UK). The throughput was fixed at 60 L/hr using a peristaltic pump (model 605DI, Watson Marlow, Falmouth, Cornwall, UK), which has previously been shown to cause no breakage to fragile particles (Clarkson, 1993). The centrifuge rotational speed was set at 12000 rpm for clarification purposes. Supernatant clarity was maintained above 95% during all trials. Prior to discharge of accumulated solid paste the feed pump was temporarily switched off and the bowl speed reduced to 8000 rpm. A partial discharge was then performed before taking samples for analytical purposes. The centrifuge was rinsed with RO water between runs in order to minimise cross-contamination.

2.2.4.3 Lab-scale centrifugation

Cells were separated from the reaction media by lab scale centrifugation for 15 minutes, 5000 rpm, 4 °C (Sorvall, Newtown, CT, USA), cells were resuspended in complex media prior to resuspension at 2g/L. The cells were then reused to assess whether they could be effectively recycled without significant cell damage. Figure 2.2 shows a comparison between the separation methods of free cells and immobilised whole cells, it is included to emphasize the comparative harshness of free cell separation.

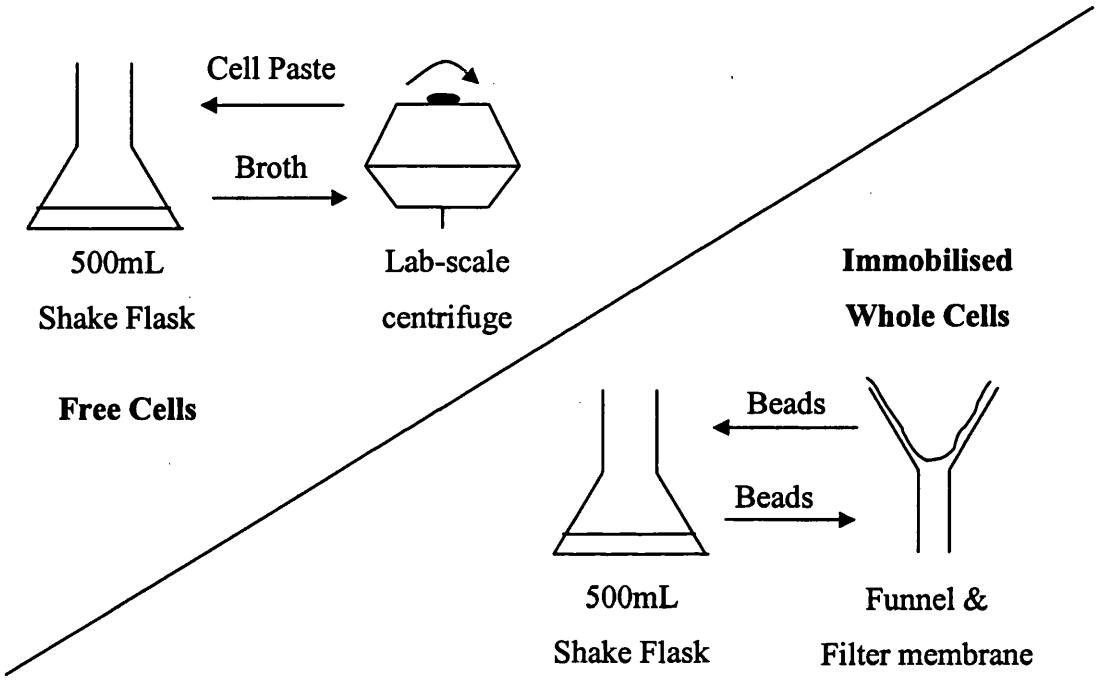


Figure 2.2: Comparison of the cell recycling methods for free cells and immobilised whole cells.

2.2.4.4 Filter cloth separation

A filter cloth was used in a funnel to manually filter the immobilised whole cell beads from the reaction media. Beads were then washed with 1 L of water prior to being resuspended in complex media and reused to assess if they could be effectively recycled.

2.2.5 Small scale Biotransformations

2.2.5.1 Isolated enzyme reaction

The consumption of NADPH is stoichiometrically linked to product formation in reactions catalysed by CHMO (Figure 2.3). NADPH absorbs strongly at 340 nm whereas NADP does not and this is the basis for the following spectrophotometric assay, which measures the activity of isolated CHMO. The method used is based on the protocol of Donoghue and Trudgill (1975).

Isolated enzyme reactions were carried out using a Uvikon 922 variable wavelength spectrophotometer (Kontron, Watford, Herts., UK) equipped with a constant temperature cell. A 1.5 mL cuvette with a 1 cm lightpath was charged with 750 µl of 50 mM Tris/HCl buffer pH 9, containing 1 mg/mL of bovine serum albumin (BSA). To this 50 µL of cell extract and then 100 µL NADPH solution in water was added and pipette mixed five times. The final concentration of NADPH in the cuvette was 0.16 mM, after addition of the substrate.

Initially the background rate of change in adsorbance at 340 nm was measured over 2 minutes at 37 °C. 100 µL of an aqueous solution of the substrate/product to be tested for isolated enzyme activity, (Figures 2.3, 2.4 and 2.5), was then quickly added to the cuvette to give the desired final substrate concentration (varied by experimental design). The consequent rate of change of adsorbance at 340 nm was measured for 2 minutes. The results of a typical spectrophotometric assay and an example calculation of the enzyme activity are given in Appendix III. Figure III.1. The reaction rate of the isolated enzyme was expressed

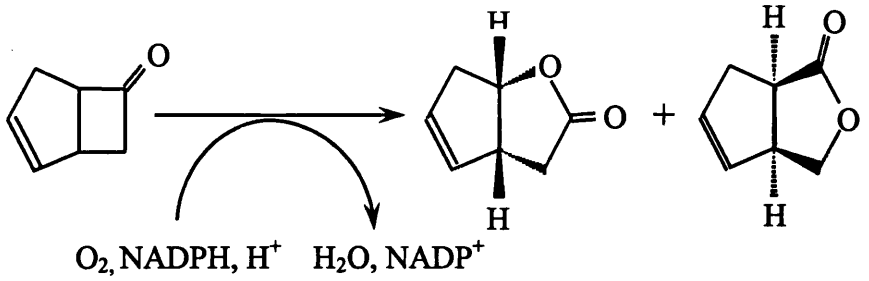


Figure 2.3: Reaction scheme of bicyclo[3,2,0]hept-6-en-2-one to its two lactone regio-isomers, (-)-(1S,5R)-2-oxa-bicyclo[3,3,0]oct-6-en-3-one and (-)-(1R,5S)-3-oxa-bicyclo[3,3,0]oct-6-en-2-one.

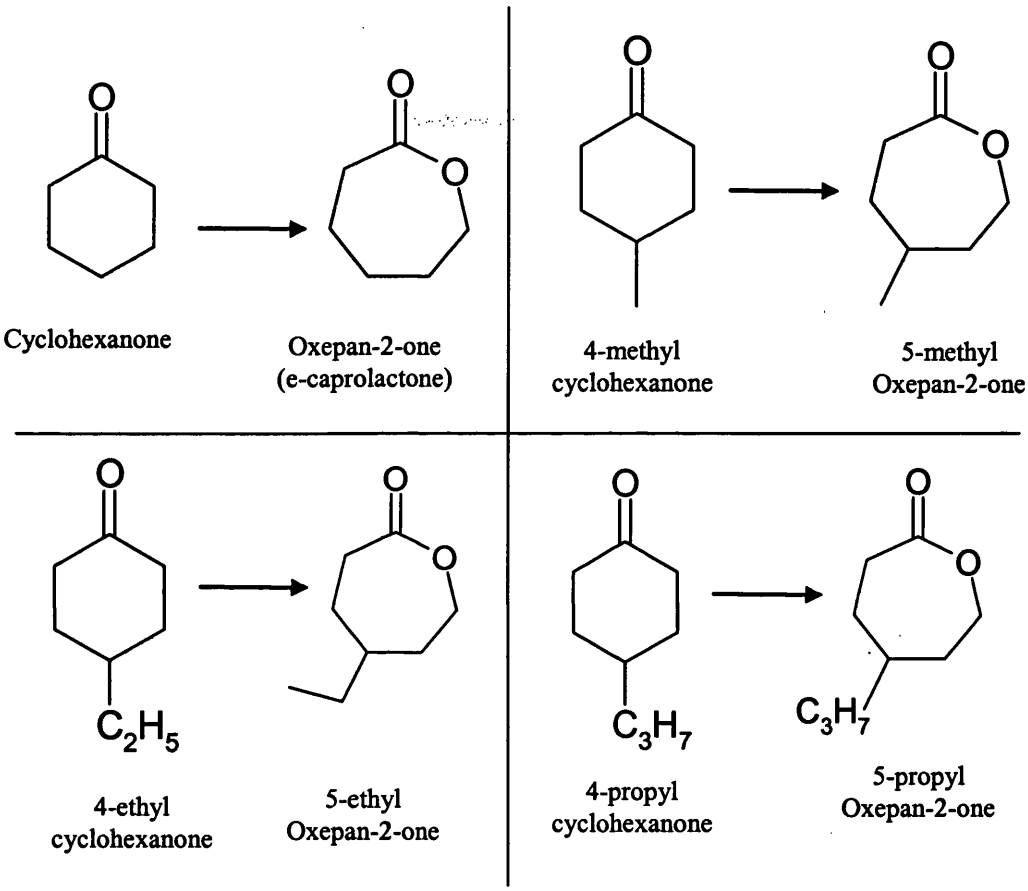


Figure 2.4: Ketones and their respective lactone structures. Similar compounds with an increasing chain length shown.

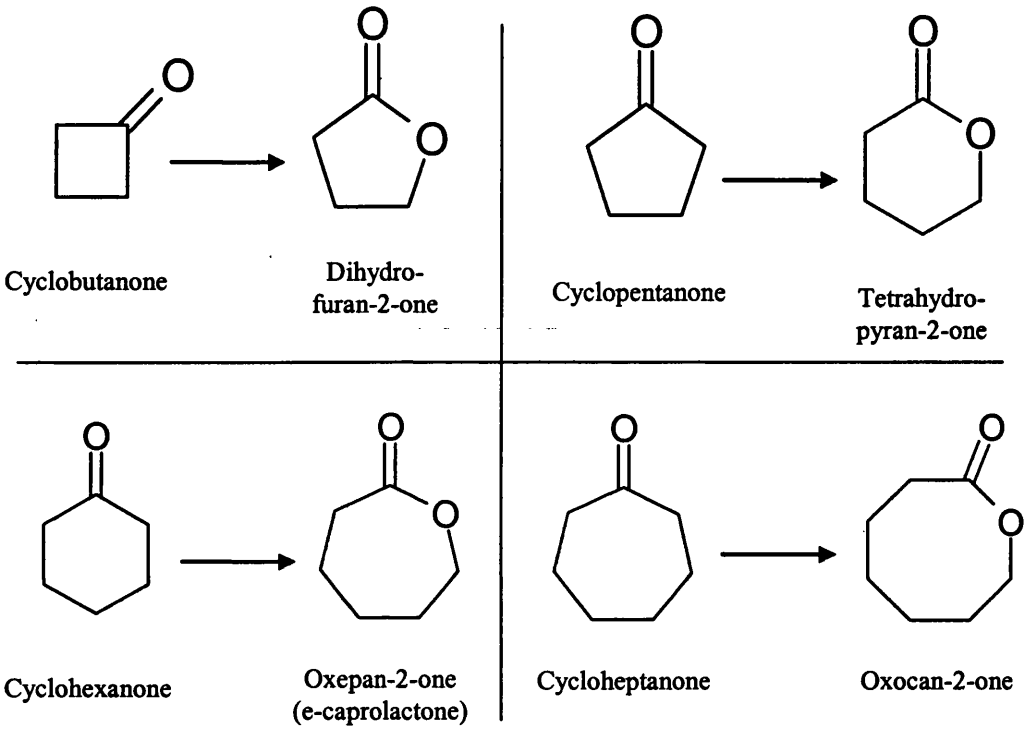


Figure 2.5: Ketones and their respective lactone structures. Similar compounds with an increasing ring size shown.

in Units (U = μmol of NADPH consumed per minute). The coefficient of variance for an individual reaction was determined from ten reactions and was found to be ± 8 %.

2.2.5.2 Whole cell and immobilised whole cell reactions

Shaken flask experiments

Biotransformations with free and immobilised whole-cells and various substrates and products (Figures 2.3, 2.4 and 2.5) at varying concentrations were studied in 500 mL (100 mL working volume) baffled shaken flasks at 37°C in the aforementioned orbital shaker at 200 rpm.

Free cell reactions of *E. coli* TOP10 and *E. coli* JM107 used the fermentation broth (the production of which is described in section 2.2.1.3), diluted with complex media to a cell density of 2g(DCW)/L to prevent oxygen limitation based on the results of Doig *et al.* (2003) and verified experimentally (Appendix IV, Figure IV.1). Reactions were studied for 1 hour with samples taken every 10 minutes for routine analysis of product concentration (section 2.2.4.3) and at the end of the reaction for the assessment of whole cell integrity by flow cytometry (section 2.2.4.3).

Immobilised whole cell reactions used 10g of beads (wet weight) suspended in 100 mL of complex media, giving a cell density equivalent to that in the free cell reactions of 2g(DCW)/L. Reactions were studied for 2 hours with samples taken every 30 minutes (Statistically designed experiments) or every 20 minutes (Subsequent experiments) for routine analysis of product concentration. In the case of the statistically designed experiments a sample of the reaction broth was taken at the end of the reaction for analysis of bead integrity (section 2.2.4.4).

The coefficient of variance on any individual initial specific free cell reaction rate using cells from a single fermentation was determined from ten repeated shake flask reactions with 2 g/L biomass and 10 mM of bicyclo[3.2.0]hept-2-en-6-one to be ±4%. The coefficient of variance on the immobilised whole cells reaction rate, determined from ten simultaneously run reactions from a single fermentation and batch of immobilised beads (see figure 5.2), was found to be ±12%.

2 L fed-batch reactions

Reactions were performed in a LH 210 series 2 L fermenter, with a 1.5 L working volume (Bioprocess Engineering Services, Charing, Kent, UK). The fermenter itself was very similar to the fermenter described in section 2.2.2.3, comprising a glass vessel with two six-bladed Rushton turbine impellers and four equally spaced baffles. There were small differences in the dimensions of the fermenter; the aspect ratio was 1.72 and the ratio of the vessel diameter to the impeller diameter was 2.47. The pH was measured by an Ingold pH probe (Ingold Messtechnik, Urdorf, Switzerland) and maintained at pH 7 (± 0.05) by the metered addition of 3M NaOH and 3M H_3PO_4. DOT was measured by a polarographic oxygen electrode (Ingold Messtechnik, Urdorf, Switzerland). The vessel was aerated with 1 vvm air via a submerged sparger, and the impeller speed was set at 800 rpm in all experiments. The temperature was maintained at 37 °C using a temperature probe and heating element (Bioprocess Engineering Services Ltd, Kent, UK). Antifoam (polypropylene glycol 2000) was added manually as necessary.

1.5 L of the harvested fermentation broth (section 2.2.2.3) containing whole cells of *E. coli* TOP10 pQR239 was transferred to the 2L fermenter immediately after cell harvest. When the medium had reached 37 °C, glycerol, the reductant for the whole cells, was added to a final concentration of 10 g/L. The ketone substrate, bicyclo[3.2.0]hept-2-en-6-one, was then pumped directly to the fermenter at a rate which maintained the substrate at a non-limiting, but non-toxic conentration (0.5-2.0 g/Lhr) from an external reservoir via a peristaltic pump (Model 205U, Watson-Marlow, Falmouth, Cornwall, UK). To follow the progress of the bioconversion and facilitate substrate feed rate control, 1 mL samples were taken for gas chromatography (GC) analysis every 30 minutes, as described in section 2.2.6.3.

2.2.6 Analytical methods

2.2.6.1 Cell concentrations

Optical density (OD) measurements at 670 nm (OD_{670}) were carried out using a Uvikon 922 variable wavelength spectrophotometer (Kontron, Watford, Herts., UK)

in order to quantify biomass concentrations. A small amount of culture medium was withdrawn from the fermenter and diluted appropriately with complex medium to give seven cell suspensions with between 0.1-1 absorbance units each. The dry cell weight of the dilutions was then calculated via an external calibration curve.

2 mL aliquots of the seven cell suspensions were added to predried and preweighed 2.2mL Eppendorf tubes and the tubes were then centrifuged at 13000 rpm for two minutes. The supernatant was discarded and the process was repeated for a further 2 ml aliquot and then a 1 mL aliquot in the same vial. After the final supernatant was discarded, the tubes were dried in an oven at 100 °C until they achieved a constant weight. The dry cell weight (d wt) of the culture could then be calculated.

Calibration curves linking the OD_{670} measurement to the dry cell weight measurement were then constructed for both strains of *E. coli*, the results of which (Appendix V, Figure V.1 and V.2) gave the following equations:

Dry cell weight *E. coli* TOP10 [pQR239] = 0.52 x OD_{670}
Dry cell weight *E. coli* JM107 = 0.51 x OD_{670}

All the biomass concentrations subsequently reported in this thesis are on a dry cell weight basis.

2.2.6.2 Ketone and lactone quantification

2.2.6.2.1 Chemical synthesis of lactone products

To enable GC quantification of the lactone concentrations, when they were not commercially available they first had to be chemically synthesized. The substrates and respective lactones are given in Figures 2.3, 2.4 and 2.5.

Synthesis of Dihydro-furan-2-one and Tetrahydro-pyran-2-one

Tetrahydrofuran (860 μL) was added to a round bottomed flask containing acetone (10 mL), potassium permanganate (1.6 g, 1.0 mmol) and iron (III) chloride (1.0 g, 6.2

mmol). The mixture was cooled to –78 °C and stirred. After two hours the cooling bath was removed and allowed to warm up to room temperature and stirred for a further sixteen hours. Dichloromethane (20 mL) was added to the reaction mixture and filtered, decolourised over charcoal, dried over $MgSO_4$ and vacuum dried to yield a colourless oily product.

Tetrahydro-pyran-2-one was synthesised as above starting from tetrahydropyran. (Lai and Lee, 2002).

Synthesis of oxocan-2-one

Oxocan-2-one was synthesised following procedures according to Bidd and co workers (Bidd *et al.*, 1983).

Dichloromethane (6.4 mL) was added to acetic anhydride (5 mL) in a cooled reaction flask fitted with a condenser. Hydrogen peroxide (30%, 4 mL) was added drop wise over thirty minutes after which maleic anhydride (4 g) was added and stirred with cooling. After one hour cooling was removed and the reaction allowed to heat up to reflux. After no more spontaneous reflux was visible (approx two hours) the reaction mixture was left to cool down to room temperature (approx two hours). Cycloheptanone (1 mL) was added and gentle reflux was maintained for fifteen hours after which the mixture was cooled and filtered to remove the maleic acid. The filtrate was then washed with water (three washes), 10% potassium hydroxide and 10% sodium sulphite (three washes) followed by water (three washes). The resulting mixture was dried over $MgSO_4$ and evaporated to yield the lactone.

Synthesis of di-substituted cyclohexanones

mCPBA (2.07 g, 12 mM) and $NaHCO_3$ (1.008 g, 12 mM) were added to dry dichloromethane (50 mL) and stirred under argon for one hour. 500 µL ketones (4-methylcyclohexanone, 4-ethylcyclohexanone and 4-propylcyclohexanon) were added and stirred for sixteen hours at room temperature. Once complete the reaction was neutralised with 10% $NaCO_3$ (three washes), brine (three washes), dried over $MgSO_4$ and concentrated under reduced pressure to yield the final lactone products. (Jagt *et al.*, 2001).

2.2.6.2.2 Sample preparation for GC analysis

Aqueous samples were prepared and analysed for ketone and lactone concentration analysis in the following way:
1 mL aqueous sample (without prior removal of cells) and 1 mL ethyl acetate with 1% naphthalene internal standard were vortex mixed for thirty seconds in a 2.2 mL Eppendorf tube. This was determined to be long enough for extraction equilibrium to be reached (data not shown). The sample was then centrifuged at 13000 rpm for one minute (Biofuge 13, Heraeus Sepatech, Brentwood, Essex, UK) and the top solvent layer was transferred into a separate vial and analysed by GC, as described in section 2.2.6.3.3.

2.2.6.2.3 GC operation

Detection and quantification of the ketone substrate and lactone product concentrations were performed simultaneously using a Perkin-Elmer autosystem XL-2 gas chromatograph, (Perkin-Elmer, Norwark, CT, USA), fitted with a ZP1 non-polar dimethylsiloxane column (30 m × 25 mm × 25 μm) with helium as the mobile phase (Phenomenex, Macclesfield, Cheshire, UK). Sample of 1 μL were injected onto the column using the integrated autosampler and compounds exiting the column were detected by a flame ionisation detector (FID). The GC operating temperatures were set as follows: column 110 °C, injector 250 °C and detector 250 °C. Data capture and analysis was achieved using Perkin-Elmer Nelson Turbochrom™ software.

Chiral analysis was performed on the same GC, with 1 μL samples injected onto a Chiraldex-B column (25 m × 25 mm × 25 μm) with helium as the mobile phase (SGE, Milton Keynes, Bucks., UK) with operating temperatures set as follows: column 90 °C, held for four mins followed by ramp at 15° C/min to 120 °C, injector 200 °C and detector 200 °C.

2.2.6.2.4 Quantification of the GC response

The FID response to a particular solute concentration in a sample was measured as an integrated peak area on a GC chromatogram. External calibration curves (concentration range 0.05-3 g/L) were used to quantify these responses for the ketone substrates and lactone products, which were prepared in complex media and extracted into ethyl acetate with the naphthalene internal standard in the same way as reaction samples.

Enantiomeric excess (ee) was calculated as:

$$ee = \frac{A - B}{A + B} \times 100\%$$

Where:

A = proportion of the more abundant enantiomer

B = proportion of the less abundant enantiomer

2.2.6.3 Immobilised whole cell bead integrity

All bead integrities of immobilised cells reported were calculated on a dry mass basis from the increase in mass in pre-weighed eppendorfs. 2 mL liquid samples were centrifuged and the pellet dried in an oven at 100 °C until a constant mass, recorded in the same way as dry cell weight measurements, was calculated (section 2.2.4.1).

2.2.6.4 Whole cell viability

Flow cytometric analyses were conducted using an EPICS XL-MCL (autoloader) 4 Colour Bench Top Flow Cytometer with Flow Centre II Acquisition Workstation, with 488nm excitation from an argon-ion laser at 15mW (Beckman Coulter, High Wycombe, Bucks., U.K.). All analytical solutions were filtered prior to use using a 0.2mm filter (Pall, Portsmouth, Hants., UK) to remove any particulate contamination that could interfere with analysis. Culture samples were diluted in Dulbecco's Phosphate buffered saline, pH 7.2 (DBS) to minimise cell aggregation and stained

with propidium iodide (PI) and bis-(1,3-dibutylbarbituric acid)trimethine oxonol (BOX) at a final concentration of 5 and 10mg/ml respectively following the method developed by Hewitt and Nebe-Von-Caron (2001). PI and BOX fluorescence were measured at 630 and 525nm respectively following a 10min incubation period at room temperature. A total of fifty thousand cells were analysed per sample. The coefficient of variance based on five repeated flow cytometric analyses was ±1%. Figure 2.6 shows the comparative different gated regions which indicate the physiological state of the cells.

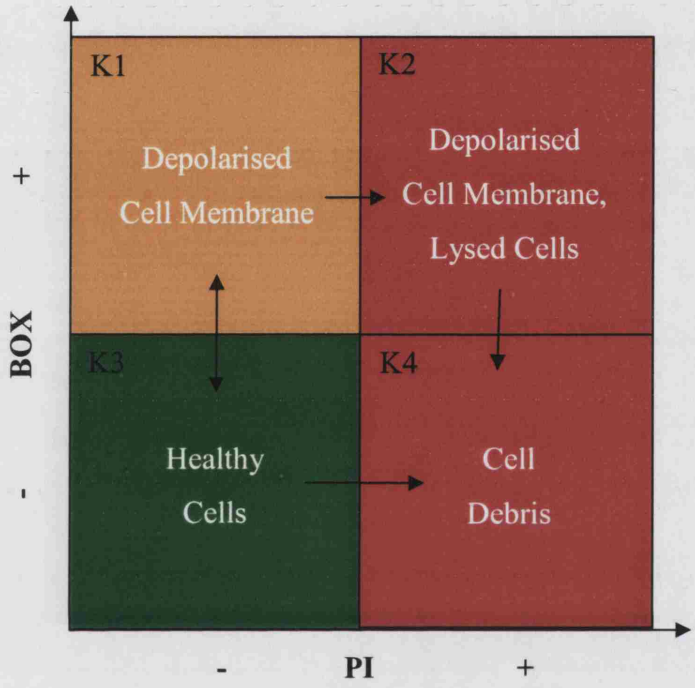


Figure 2.6: Flow cytometry gated quadrants. Healthy cells (K3) are unstained, cells with no membrane potential (K1) are stained with BOX, cells with permeabilised membranes (K2) are stained with BOX and PI and other cells fragment (K4) are stained with PI.

RESULTS & DISCUSSION

2.3 Enzyme host comparison

2.3.1 Available host cells

Whilst microbiology and gene cloning does not form part of this project, it is important that prior to commencing further studies that the best available cell host into which CHMO had been cloned at UCL was selected. There were two cell hosts available for use within this study, both being strains of *E. coli*:

1. **TOP10 [pQR239]** – this system has been recently developed and is considered the superior host due to over-expression of the enzyme (twenty five times higher protein concentration than found in the wild type and JM107), which is induced using the cheap (L)-arabinose promoter (Doig *et al.*, 2001a).
2. **JM107** – this cell host had previously been written off due to the CHMO enzyme not being over-expressed, i.e. only comparable amounts of CHMO being present as that in the wild type host (Chen *et al.*, 1988). Enzyme expression is induced using IPTG. However for whole cell biocatalysis it is known that the enzyme is present in excess (i.e. by comparison of the reaction rate seen in whole cells to that of isolated enzyme activity). As whole cell biocatalysis is preferable to the use of isolated enzymes, see figure 1.4, the over-expressed CHMO enzyme is less important than enzyme stability and the degree of substrate/product inhibition exhibited if the enzyme concentration is not reaction rate limiting.

To assess the different host cells, under conditions set out in section 2.2.5.2, the following have been compared:

- The effect of substrate and product inhibition using bicyclo[3.2.0]hept-2-en-6-one and its corresponding lactone product as a test reaction system as has been commonly employed previously (section 1.2.2.2).
- The stability of CHMO in the two *E. coli* hosts.

- The effect of the 25-fold difference in enzyme concentration on the whole free cell reaction rate.

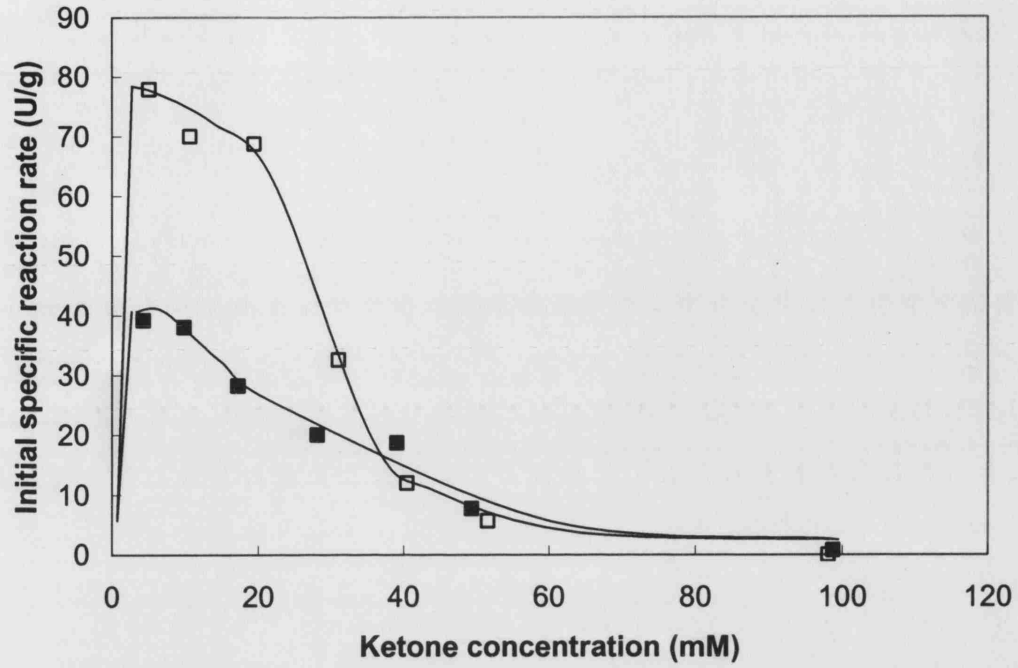
2.3.2 Effect of inhibition on the two host cells

The inhibition of the two *E. coli* strains, as studied in the non-oxygen limited shaken flask reaction system described in section 2.2.5.2, over a range of substrate and product concentrations is shown in Figure 2.7.

The JM107 strain, in which the enzyme is not over-expressed, gave a specific reaction rate approximately half of that in the TOP10. As the CHMO expressed in TOP10 was found to be twenty five-fold higher than that in JM107, the twofold increase in reaction rate observed suggests that the enzyme is not reaction rate limiting in TOP10. Doig *et al.* (2003) believed that substrate/product diffusion through the cell membrane was the rate limiting step.

Excluding the reduced reaction rate, the effect of substrate concentration on JM107 appears to yield a similar substrate inhibition profile to that of TOP10 when oxygen is not reaction rate limiting. Whilst the product inhibition profiles are also similar, at a lactone concentration of 40mM significant product inhibition was apparent with JM107 (negligible reaction rate), whereas with TOP10 it was not. The higher level of inhibition seen within the JM107 system at this product concentration, suggests that product inhibition is also mildly dependent upon the intracellular concentration of CHMO.

A major constraint on the rate of reaction observed within CHMO biocatalysis, as identified in Figure 1.5, is oxygen availability. However, a high oxygen concentration (above nil% DOT) is known to be deleterious to CHMO activity during the fermentation (Doig *et al.*, 2001a). This is potentially a cause of the low enzyme stability (enzyme activity for a batch of cells was maintained for only 10h in non-inhibitory conditions) seen in the high oxygenation bubble reactor used by Hilker *et al.* (2004a) in conjunction with ISSSPR (see section 1.2.2.3). To assess the effect of



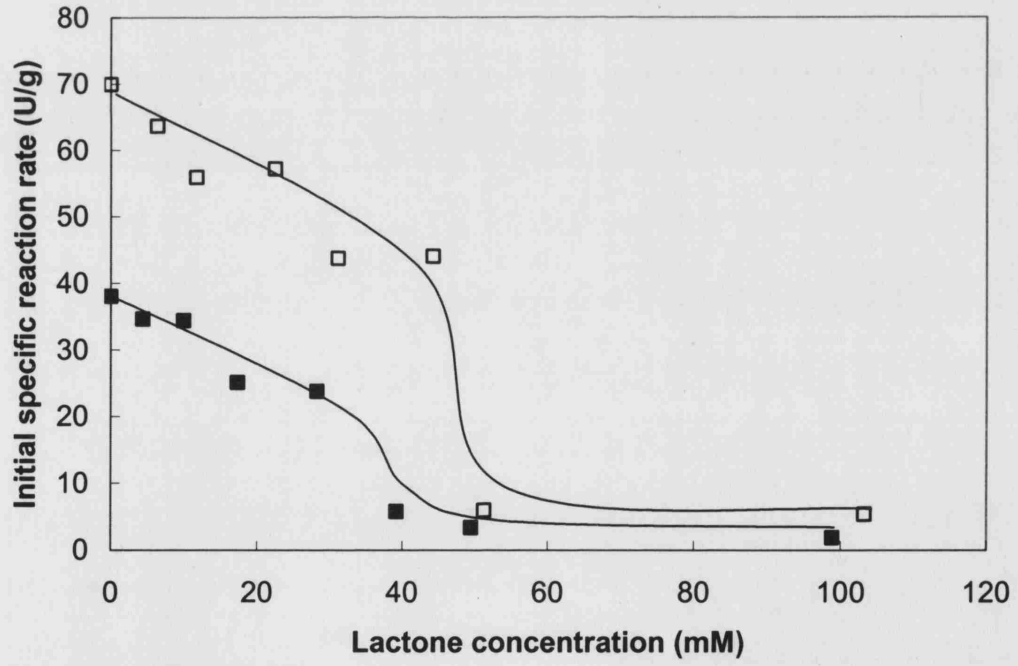


Figure 2.7: Comparison between the substrate *(TOP)* and product *(BOTTOM)* inhibition of CHMO expressed in *E. coli* with various bicyclo[3.2.0]hept-2-en-6-one and (-) 1(R), 5(S) 2-oxabicyclo[3.3.0]oct-6-en-3-one concentrations (with 5mM ketone).

□ TOP10 [pQR239], ■ JM107

product inhibition over a longer reaction time, with oxygen held at a concentration which is reaction rate limiting (nil% DOT), i.e. to remove the potentially deleterious effect of high oxygen concentrations used in shaken flask experiments, both *E. coli* strains were studied in a fed-batch bioconversion of bicyclo[3.2.0]hept-2-en-6-one in a 2L fermenter. The reaction progress was monitored and the pump rate was adjusted to maintain a ketone concentration below 10mM (i.e. below the level where significant substrate inhibition was observed above) whilst not allowing it to become rate limiting.

The results of these experiments are shown in Figure 2.8. The reaction rate of JM107 (14.7 U/g) was higher than that of TOP10 (12.5 U/g) at a cell density of 8.5 g(DCW)/L. Therefore under oxygen limited conditions the JM107 strain appears to be more oxygen efficient.

It is worth noting that this CHMO bioconversion has a relatively poor oxygen utilisation efficiency in *E. coli* TOP10, with almost seven moles of O_2 being required to produce every mole of lactone (Doig *et al.*, 2003); the excess being used by the cells for other metabolic processes. Therefore as has been recently concluded by Baldwin *et al.* (in press) the cell densities used in reactions under oxygen limited conditions may in fact reduce the actual reaction rate.

For both strains at the larger scale (Figure 2.8), the final concentration of lactone produced was significantly lower than that at which inhibition was seen in the shorter length shaken flask experiments (e.g. around 25mM compared to 30-40mM for JM107). As this reaction in TOP10 has been previously demonstrated to be scaleable, both upwards and downwards (Doig *et al.*, 2002a and 2002b respectively), it appears reasonable to conclude that the inhibitory effect of the substrate and product are also time dependent. Supporting this hypothesis is the result that even at high concentrations of substrate (100mM), a small amount of product was formed in the first ten minutes, prior to further reaction being completely inhibited.

It has not escaped notice that this time based inhibition could possibly be the reason that only sixteen hours of enzyme activity was seen with the TOP10 host strain when

ISPR was employed to prevent the initial substrate and produce inhibitory concentrations being reached (Simpson *et al.*, 2001). The potential influence of time on inhibition appears interesting. Unfortunately attempts to utilise Near Infrared Spectroscopy (results not shown) as described by Bird *et al.* (2002) for faster control over substrate and product concentrations still proved too slow for a reasonable investigation into this phenomenon. Therefore further studies investigated only the effect of inhibition on the initial reaction rate.

Whilst the predominant difference between the two strains from these data is the increased specific reaction rate of TOP10 under non oxygen limited conditions, a further advantage seen was that TOP10 can maintain its enzyme stability when stored in the fridge for up to a week (Walton and Stewart, 2002) whereas the JM107 strain maintained only a small amount of activity after twenty four hours and no visible activity after forty eight hours (results not shown). A potential reason for this phenomenon with similar *E. coli* hosts being the twenty five-fold over-expression of CHMO in TOP10 compared to JM107.

The stability of cells has been shown to be improved by immobilisation. For example Gandolfi *et al.* (2004) entrapped *Acetobacter aceti* in calcium alginate for the oxidation of 2-phenylethanol and found that immobilised cells had higher specific activity, substrate tolerance and stability. To determine whether similar effects of immobilisation are exhibited in this system, immobilised *E. coli* cells were assessed.

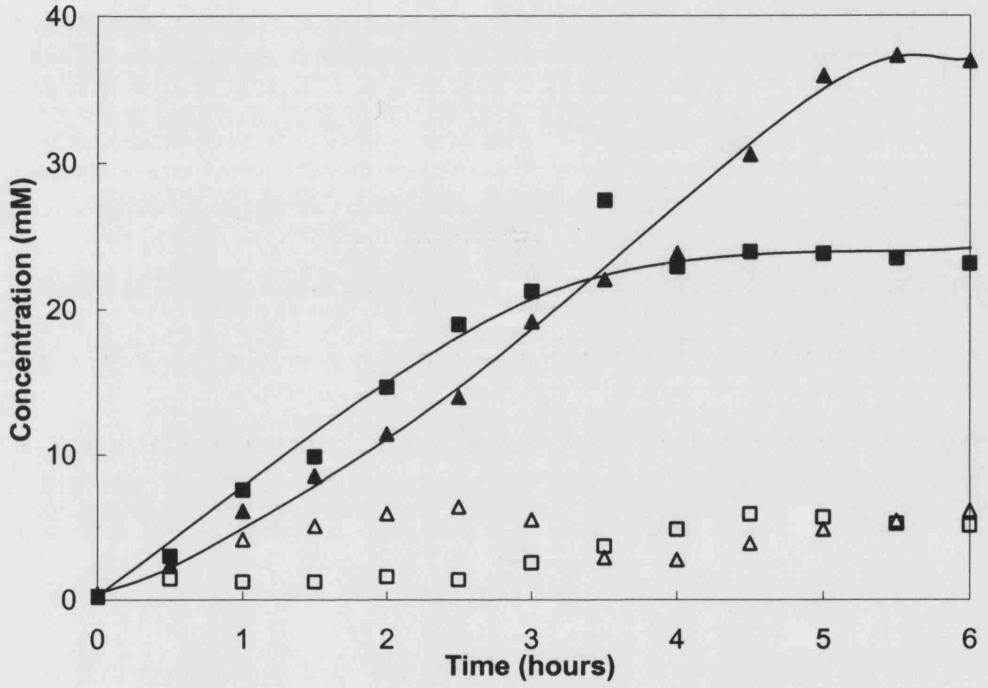


Figure 2.8: Time course comparison of *E. coli* TOP10 [pQR239] and *E. coli* JM107 catalysed reactions of bicyclo[3,2,0]hept-6-en-2-one in a 2L fermenter.

JM107: □Ketone, ■ Lactone

TOP10:△Ketone, ▲ Lactone

2.4 Whole cell immobilisation

2.4.1 Statistically designed whole cell immobilisation

Many reports of the use of calcium alginate immobilisation have been published with significantly varied immobilised conditions being employed. However, conventional methods based on changing one factor at a time fail to reveal any potential interactions between factors. The widening use of statistical design of experiments comes as more researchers become aware of the benefits of such studies:

- The most efficient use of resources – every individual experimental range of factors being incorporated, yet the experimental space that can be studied is larger than possible in so few traditional experiments (Serralha *et al.*, 2004).
- Not only are the effects of single factors evaluated, but also their interactions (Annadurai *et al.*, 2002).
- If coupled with high-throughput scale-down experiments information can be rapidly gathered (Mount *et al.*, 2003).

Calcium alginate immobilisation of E. coli TOP10 [pQR239] was employed with the aim of increasing the enzyme stability, hopefully making the cells less prone to inhibition and easier to recycle or potentially integrate with chemical synthesis steps in pharmaceutical development. As the assessment of the effect of immobilisation was to be carried out in baffled shaken flasks to maximise the oxygen concentration, highly ductile beads were required to prevent bead breakage. However the importance in the observed reaction rate on the volumetric productivity of the system makes this response of equal importance.

The conditions employed in the calcium alginate immobilisation process are significantly varied and find use in a wide range of applications (see Table 2.2). Due to the different conditions possible, beads with widely different properties are possible, for example the compression modulus can vary from less than 1kPa to over 1000kPa, whilst the shear modulus can vary between 0.2-40kPa (Drury *et al.*, 2004). To enable an optimised immobilised bead for the required application, statistically

designed experiments were performed to allow comparison of different factors against their responses.

As the use of factorial design of experiments is now a commonly employed technique, this study does not aim to assess the potential of this tool for the calcium alginate immobilisation of whole cells, as this has already been done elsewhere, for example Becerra *et al.* (2001) studied the effects of $CaCl_2$ concentration, bead diameter and hardening time (the relatively high conditions studied are shown in Table 2.2). Instead this study employs the tool of factorial design to locate the best operating conditions for use in subsequent experiments. Montgomery (2001) gives a full description of the potential and application of factorial design and surface response methodology.

Sodium alginate Conc. (%)	$CaCl_2$ Conc. (M)	Fridge Hardening time (h)	Cells immobilised	Use	Ref
1-4	0.18	2	*Aspergillus niger*	Production of polymethylgalactu-ronase	Angelova *et al.* (1998)
4	0.18	0.5	*Pseudomonas putida*	Reduction of thiophene	Fernandes *et al.* (2002)
1.8	0.1	1.5	*Bacillus megaterium*	Hydroxylation of hydrocarbons	Adam *et al.* (2001)
0.5-2.5	0.1	3	*Acetobacter aceti*	Oxidation of 2-phenylethanol	Gandolfi *et al.* (2004)
2	0.09	1 (30 C)	*Pseudomonas putida*	Phenol degradation	Chung *et al.* (2003)
2.1	0.27	0.5	*Serratia marcescens*	Comparison of alginate models	Laca *et al.* (2000)
2	0.2	2	*Saccharomyces cerevisiae*	3-oxo ester reduction	Buque *et al.* (2002)
0.5-1	0.1-0.5	1	*Rhodosporidium toruloides*	Kinetic resolution of 1,2-epoxyoctane	Maritz *et al.* (2003)
0.5	0.45-1.35	2-8	*Kluyveromyces lactis*	Lactose bioconversion	Becerra *et al.* (2001)
2.3	0.05-0.5	10-120 min	*Nicotiana tabacum*	Cell growth	Shibasaki-Kitakawa *et al.* (2000)

Table 2.2: Published calcium alginate immobilisation conditions, the type of cells immobilised and their uses.

From the range of calcium alginate entrapment conditions seen in Table 2.2, the factors studied in the statistically designed experiments were formulated and are given in Table 2.3. These factors were used for a variety of reasons:

- Angelova *et al.* (1998) found that alginate concentrations of 1% were weak and often fragmented whilst concentrations of 2, 3 and 4% gave "enduring consistency".
- Calcium chloride range selected to give a wide range of concentrations encompassing the majority of those used elsewhere (Table 2.2)
- Cell concentration range selected so that 10g of beads in a 100mL shaken flask will give both higher and lower cell densities compared to free cell equivalent, with a bias towards lower cell concentrations to enable the same concentration as used in the specific free cell reactions (i.e. 2 g/L).
- Shibasaki-Kitakawa *et al.* (2000) found that beads became brittle with increased bead hardening time in $CaCl_2$, hence a lower end range of this factor was studied.
- Becerra et al. (2001) studied relatively high ranges of $CaCl_2$ and fridge hardening times and found the lowest concentration of calcium chloride and shortened fridge hardening times yielded the highest enzyme activity for small beads, therefore this study attempted to focus on comparatively low ranges.

Factors	Ranges	Responses
Sodium alginate concentration	2-4% w/v	Reaction rate (μM/min)
Calcium chloride concentration	0.05-0.95M	% bead integrity
Cell concentration	0.01-0.13g(DCW)/gbead	
Fridge hardening time	0-30 minutes	

Table 2.3: Statistical design factors, their ranges and the responses studied.

2.4.2 Selection of immobilisation conditions

A previously reported attempt to employ calcium alginate immobilised beads to reduce the level of inhibition showed that the bioconversion of bicyclo[3.2.0]hept-2-en-6-one was both very slow (unreported reaction rate) and only 2g/L of lactone was produced (Simpson *et al.*, 2001). Ostberg *et al.* (1993) found that high gelling times caused a retardation in the release of the drug they immobilised in calcium alginate, thus it appears reasonable to suggest that the long (sixteen hour) fridge hardening time employed by Simpson *et al.* (2001) is likely to have significantly reduced the mass transfer rate and thus the reaction rate and may have damaged the CHMO activity.

The ANOVA (Analysis of variance) test of the data in this study showed that there was no statistically important individual factor and the R^2 value of >98% suggested that this was a statistically viable result. Interestingly this shows that over the tenfold difference studied, cell concentration had no effect on the rate of reaction (See Figure 2.9 for confirmation). A similar result was presented by Maritz *et al.* (2003) who found that the immobilised biomass concentration had no effect on the apparent activity of *Rhodosporidium toruloides.* Whilst no individual factor was recognised as significant in this study, other studies with different systems have found some factors to have an effect. For example, Maritz *et al.* (2003) found a marginal effect of higher alginate concentrations reducing the observed enzyme activity. A potential reason for the similar reaction rate seen with different conditions could be that the diffusion limitation caused by the immobilisation is constant for each type of bead and thus the available oxygen is used by only the outermost cells (i.e. those with access to it) to perform the bioconversion. A noteworthy point which can be drawn from this study is that the optimum calcium alginate immobilisation conditions used can be highly tailored to suit the bead properties required.

The responses to the factorial experiments (Figure 2.9) show that four points lie outside the main cluster of other points; these are the key results and show the significant interactions (factors and responses highlighted in Table 2.4). In summary two sets of three factor interactions (4%w/w sodium alginate, 0.95M $CaCl_2$, 30 minutes fridge time and 2%w/w sodium alginate, 0.05 M $CaCl_2$, 30 minutes fridge

time – effects of cell concentration having no effect) were observed which were detrimental to the bead integrity and thus led to an increase in free cells and hence apparent reaction rate. Without statistical experimental design these negative interactions would not have been found.

Following this result, the following conditions were selected for further experimentation: 2% w/v sodium alginate, 0.95M calcium chloride and 0 minutes in hardening solution in the fridge. These conditions were selected due to the relative ease of producing the lower concentration of sodium alginate, the strength of calcium chloride ensuring strong calcium alginate bead formation and the lack of fridge hardening time reflecting the negative impact of this in the three factor interactions noted in the statistically designed experiments. Such conditions resulted in a reaction rate of 34 U/g and bead integrity of 99%, as shown in Figure 2.9.

As the statistical design showed that cell concentration had no effect on the strength of the beads formed, this was independently set to allow comparison between free and immobilised cells. A cell density of 2g(DCW)/L was used in the standard free cell reactions (section 2.2.5.2), thus the same amount of cells were used to produce 10g (wet weight) of immobilised beads used in a standard immobilised cell reaction, this being equivalent to 0.02g(DCW) cells/g bead.

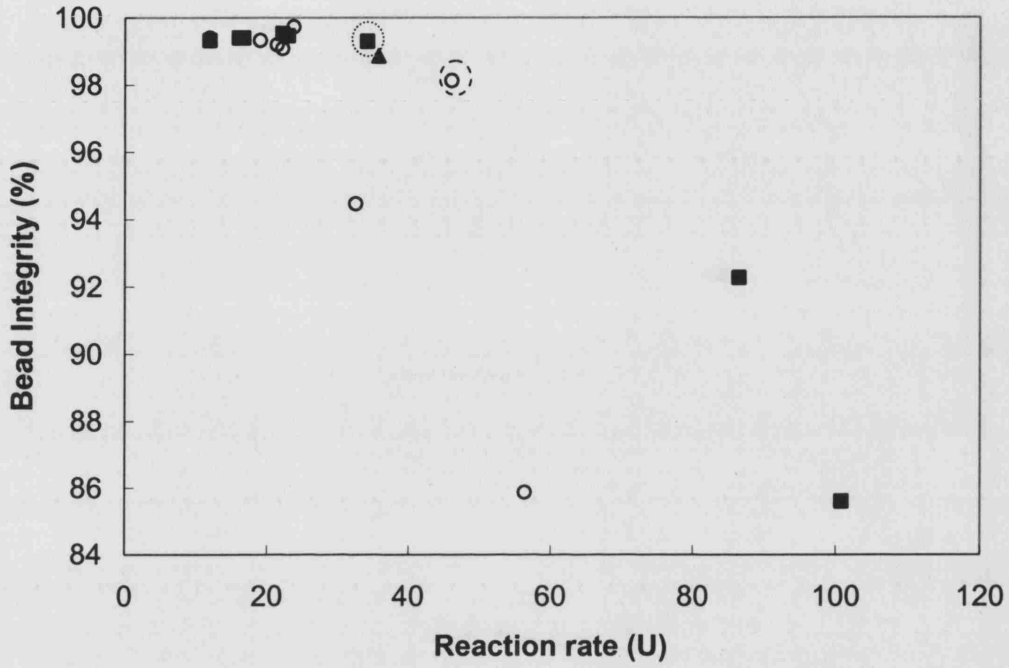


Figure 2.9: Statistically designed experiment results, bead integrity and reaction rate for the levels of different factors studied: sodium alginate concentration (2 – 4 % w/v), calcium chloride (0.05 – 0.95 M), time in fridge in hardening solution (0 – 30 minutes), cell concentration (0.01 – 0.13 g(DCW)/g bead).

Where:

Cells concentrations of ○ 0.01g/g, ▲ 0.07g/g and ■ 0.14g/g are separately illustrated. Examples of conditions are circled:

◌ 0.13g (DCW) cells/g bead, 2% w/v sodium alginate, 0.95M calcium chloride, 0 minutes in hardening solution in fridge.

◌ 0.01g (DCW) cells/g bead, 4% w/v sodium alginate, 0.05M calcium chloride, 0 minutes in hardening solution in fridge.

Cell Concentration (g(DCW)/g bead)	Sodium alginate Concentration (% w/v)	$CaCl_2$ Concentration (M)	Fridge hardening time (minutes)	**Reaction rate (mM/min)**	**Bead integrity (%)**
0.1	2	0.05	0	**21**	**99.2**
0.1	4	0.05	0	**46**	**98.1**
0.1	2	0.95	0	**22**	**99.1**
0.1	4	0.95	0	**12**	**99.4**
0.1	2	0.05	30	**33**	**94.5**
0.1	4	0.05	30	**24**	**99.7**
0.1	2	0.95	30	**19**	**99.3**
0.1	4	0.95	30	**56**	**85.9**
0.7	3	0.5	15	**36**	**98.9**
1.3	2	0.05	0	**23**	**99.5**
1.3	4	0.05	0	**16**	**99.4**
1.3	2	0.95	0	**34**	**99.3**
1.3	4	0.95	0	**12**	**99.3**
1.3	2	0.05	30	**87**	**92.3**
1.3	4	0.05	30	**17**	**99.4**
1.3	2	0.95	30	**22**	**99.5**
1.3	4	0.95	30	**101**	**85.6**

Table 2.4: Results of the factorial design experiments. The four factors studied are shown in the leftmost columns and their responses in bold to the right. Rows highlighted show the three factor interactions which gave poor bead integrities.

The two three-factor interactions identified that yielded less bead integrity were of either high alginate and $CaCl_2$ concentration or low concentrations of both, with high fridge hardening times. Becerra *et al.* (2001) found a similar result, based on their three factor statistically designed experiments (alginate concentration not studied) on calcium alginate entrapment. They found that either a high strength $CaCl_2$ and low fridge hardening time or a low $CaCl_2$ strength and a high fridge hardening time gave the most stable enzyme activity (note ranges studied were higher than those reported here), i.e. high and high and low and low were less suitable. Given the relatively low $CaCl_2$ concentration range studied here, this result was not observed.

Maritz *et al.* (2003) found that increasing the alginate concentration led to increased particle sizes, with 0.5% w/v alginate producing beads of around 2mm and 1% w/v alginate producing beads of around 2.5mm. They concluded that this was due to the increased viscosity. The effect of potentially differing bead diameters produced during the factorial design was not included in this study, however no visible bead size differences were seen with the higher alginate concentrations studied (2-4% w/v) and the individual factor of alginate concentration appeared to have no effect on the reaction rate. The effect of bead size was studied individually in section 2.5.4.2.

For the low cell concentration (equivalent to 1 g/L DCW) with lowest bead integrity (86%), the reaction rate of 56 U/g was observed. This is approaching the specific free cell reaction rate shown in Figure 2.7 (70 U/g), which suggests that although the bead integrity remains relatively high, the majority of cells have been released from the alginate beads and thus bead integrity is of paramount importance if reuse, simple separation and integration with chemical synthesis steps are desired. Attempts to corroborate this conclusion, through quantification of the cell density though optical density measurement, were unsuccessful as an apparent cell density >40% higher than that of free cells was observed. This is likely to be a result of other bead breakdown products. This result also confirms that the mild calcium alginate immobilisation process is largely undamaging to the cells as has been reported elsewhere (Adam *et al.*, 2001; Buque *et al.*, 2002).

The high cell concentration with the lowest bead integrity by contrast simply shows that at these high cell concentrations the reaction is oxygen limited.

The optimal conditions sought in this study were for both high bead integrity and high reaction rates. However it is clear that these two factorial design responses conflict, with low bead integrity (i.e. high levels of free cells) increasing the rate of reaction. Bead integrity was given the most importance due to the breakdown effectively being the cause of the increased reaction rate. Based on this factor alone the optimum cell immobilisation conditions of 2% w/w sodium alginate, 0.95M calcium chloride, 0 minutes in the fridge in calcium chloride was generated from the design. As only two

three factor interactions were found to yield negative immobilisation results, a wide range of potential conditions could be used for future study, rationale behind the selection are given in section 2.2.2.3.

It has been reported elsewhere (Trelles *et al.*, 2004; Jianlong *et al.*, 1999) that the reduced rate of reaction seen with cells entrapped in calcium alginate is due to either oxygen or substrate/product diffusion. Given the oxygen intensive nature of oxidation reactions, it appears from a preliminary view that oxygen is more likely to be rate limiting in the CHMO catalysed synthesis. Potential support for this argument lies with the kinetic resolution performed by Maritz *et al.* (2003) where the reaction rate was only halved when the experiment was performed with immobilised cells, whereas in this case the reaction rate was around a tenth of that of the free cells. To analyse this further the effects of beads concentration on the specific reaction rate are studied in section 2.5.3.1.

2.4.3 Immobilised whole cell characterization

2.4.3.1 Effect of bead concentration

Statistically designed experiments used 10g beads per 100 mL shake flask experiment (100g beads/L). To assess the potential effects of oxygen limitation within the system, the concentration of beads was varied by sequential doubling between 1 to 640g beads/L. The results are shown in Figure 2.10. As the free cell reaction rate was not oxygen limited, oxygen will also be available in excess in the liquid phase of the immobilised whole cell reactions.

With no oxygen limitation, it could reasonably be expected that the specific reaction rate (based on the bead concentration rather than the cell concentration) would remain constant over all bead concentrations. However with oxygen limited reactions, doubling the bead concentration is likely to halve the specific reaction rate.

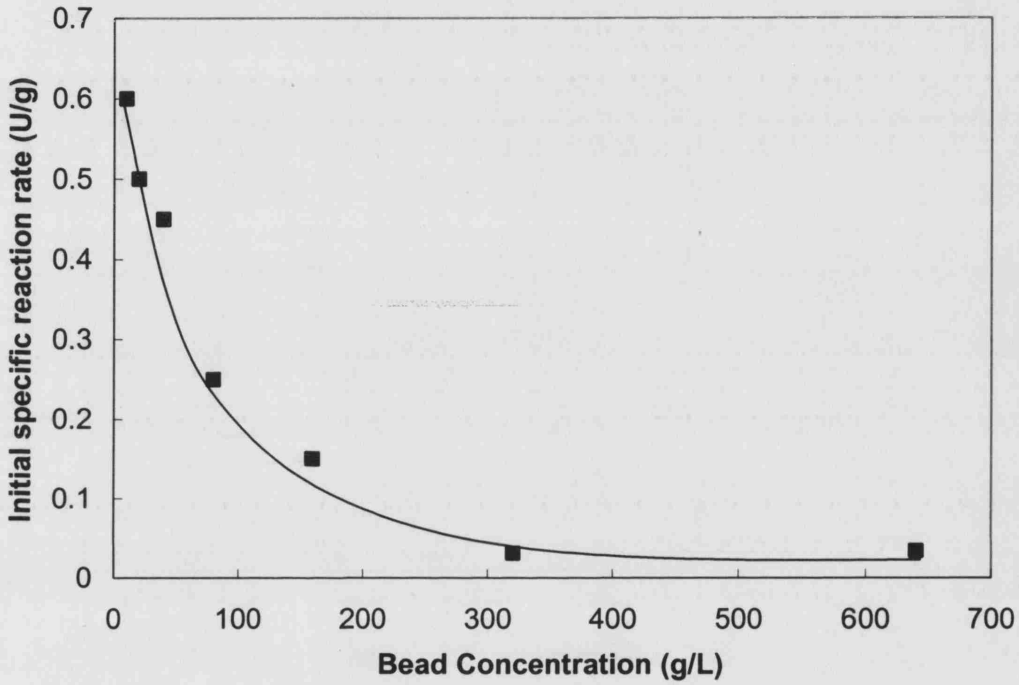


Figure 2.10: Effect of multiple doublings of the bead concentration on specific reaction rate, where the specific reaction rate is per gram of beads, not per gram of cells as for other figures.

From Figure 2.10 it appears that the immobilised whole cell reactions are both somewhat, but not wholly, oxygen limited.

For subsequent experiments a final cell concentration of 2% w/w per bead and 10g of beads was selected to allow even comparison with the free cell reaction, i.e. 0.2g of cells per 100 mL free cell reaction (2g/L DCW) and 0.2g of cells (DCW) per 10g (wet weight) of beads.

2.4.3.2 Effect of bead size

Mota *et al.* (2002) modelled the diffusion observed in reported experimental data and proposed that it was probable that the viable and reacting whole cells immobilised in alginate beads are found in a sub-surface layer. Similarly Jianlong *et al.* (1999) found that oxygen utilisation progressively increased from cells held at the core to the bead surface.

The main disadvantage of whole cell immobilisation is that it usually reduces the reaction rate (often significantly). Therefore in an attempt to increase the reaction rate of immobilised whole cells, the bead size was reduced by applying a parallel air flow as has been employed by Strand *et al.* (2002). By this method bead size could be reliably reduced from a diameter of 3mm (gravity dropped) to 1mm. Smaller beads (<0.2mm) could also be produced, though a range of sizes usually resulted due to the relative imprecision and method of size reduction used. Many size reduction techniques have been used to reduce the bead size such as rotating disc atomisation (Senuma *et al.*, 2000), electrostatic dripping (Watanabe *et al.*, 2001) and jet disruption (Serp *et al.*, 2000), however modern machined equipment using vibration for the production of monodisperse small beads have been manufactured and have reliably yielded beads as small as 0.25 mm in diameter (Serp *et al.*, 2000).

Industrial filtering characteristics have been taken to give a minimum bead size of 0.1 – 0.2mm (Kallenberg et al., 2005). A potential solution to the mass transfer limitation imposed by this minimum particle size has been recently made commercially available

by www.magneticmicrosphere.com who formulated polymers containing magnetite, which allows for immobilized cell separation by magnetic fields. It is noted that this technique is difficult at scale due to energy loss.

Figure 2.11 gives the comparative reaction profiles of free cells, large and small beads. The increase in reaction rate achieved with the 1mm immobilised beads was a two and a half-fold increase compared to their 3mm counterparts (reaction rates of 24 and 10 U/g respectively). As the tripling of the specific surface area (=3/r) has almost lead to a tripling of the reaction rate, these findings appear to support the aforementioned conclusion of Mota *et al.* (2002), that active cells appear to be in a sub-surface layer.

The bead size reduction meant that immobilised whole cells were significantly more attractive (19% of the free cell reaction rate) than gravity dropped 3mm beads (8%). Given that it is possible to produce even smaller beads reliably it appears reasonable to suggest that such sizes would yield even more comparable reaction rates. Becerra *et al.* (2001) found that at bead sizes of 0.5mm, the permeabilised immobilised whole cells were of higher activity per unit of biomass than the free cell equivalent.

From the results seen in figure 2.11, it is postulated that a bead size of 0.2 μm would be required before the reaction rate of immobilised whole cells is comparable to those of free cells. However, given that *E. coli* cells are about 2 μm in length, such a small bead size is not possible, with a minimum realistic bead diameter of 10 μm if the magnetite containing beads noted above become industrially viable.

2.4.3.3 Biocatalyst recycling

To give an impression of the stability of immobilised whole cells compared to their free cell counterparts the initial reaction and three subsequent recycles were studied (Figure 2.12) at a bicyclo[3,2,0]hept-6-en-2-one concentration of 10mM. The reaction rates achieved with both free and immobilised whole cells appears to be relatively consistent over the limited number of recycles attempted, proving that both the

laboratory scale centrifugation (also used in the calcium alginate immobilisation process) is not damaging to the reaction rate achieved.

Trelles *et al.* (2004) found that *E. coli* immobilised on agarose maintained its full activity in the synthesis of adenosine for twenty five recycles, whereas for free cells the full activity was only maintained for twelve recycles. For each recycle the equivalent product yield was observed, though at slower reaction rates for immobilised cells.

The only published limits of the CHMO activity stability in biocatalytic use have been published by Simpson *et al.* (2001) and Hilker *et al.*, (2004b) who found that when ISPR was employed the activity of the cells was zero after sixteen and ten hours of continuous use respectively. This method of assessing the CHMO stability in immobilised whole cells is a good comparative measure of enzyme stability and would exclude the potential effects of multiple centrifugation and resuspension steps. Attempts to replicate these studies with immobilised beads proved unsuccessful.

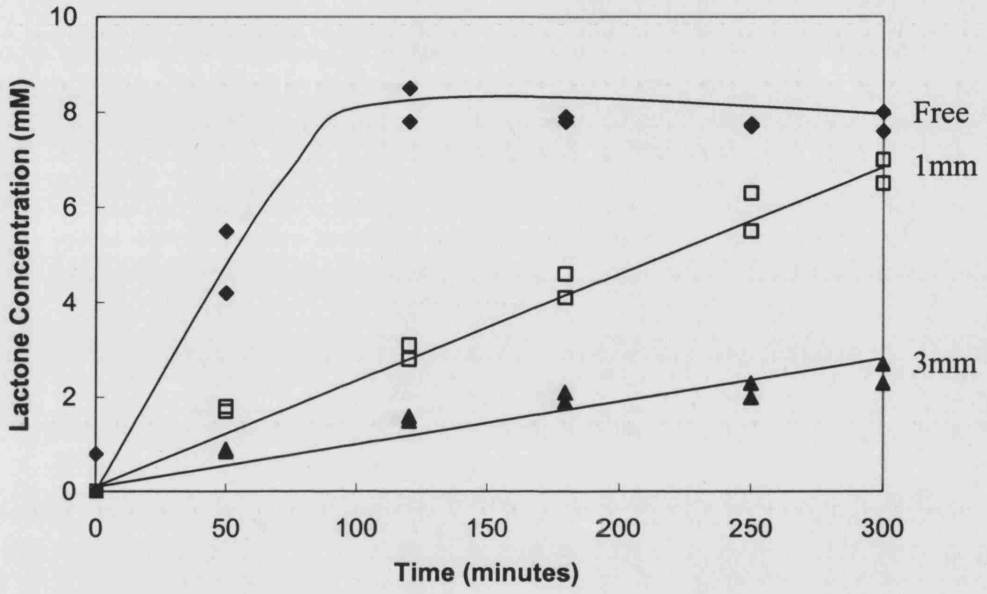


Figure 2.11: Comparison of lactone produced from duplicated results of gravity produced 3mm diameter calcium alginate immobilised cells (▲), reduced size (parallel air-flow aided) 1mm immobilised cells (□) and free cells (◆). The actual dry weight concentration of cells was constant at 2 g/L in all experiments.

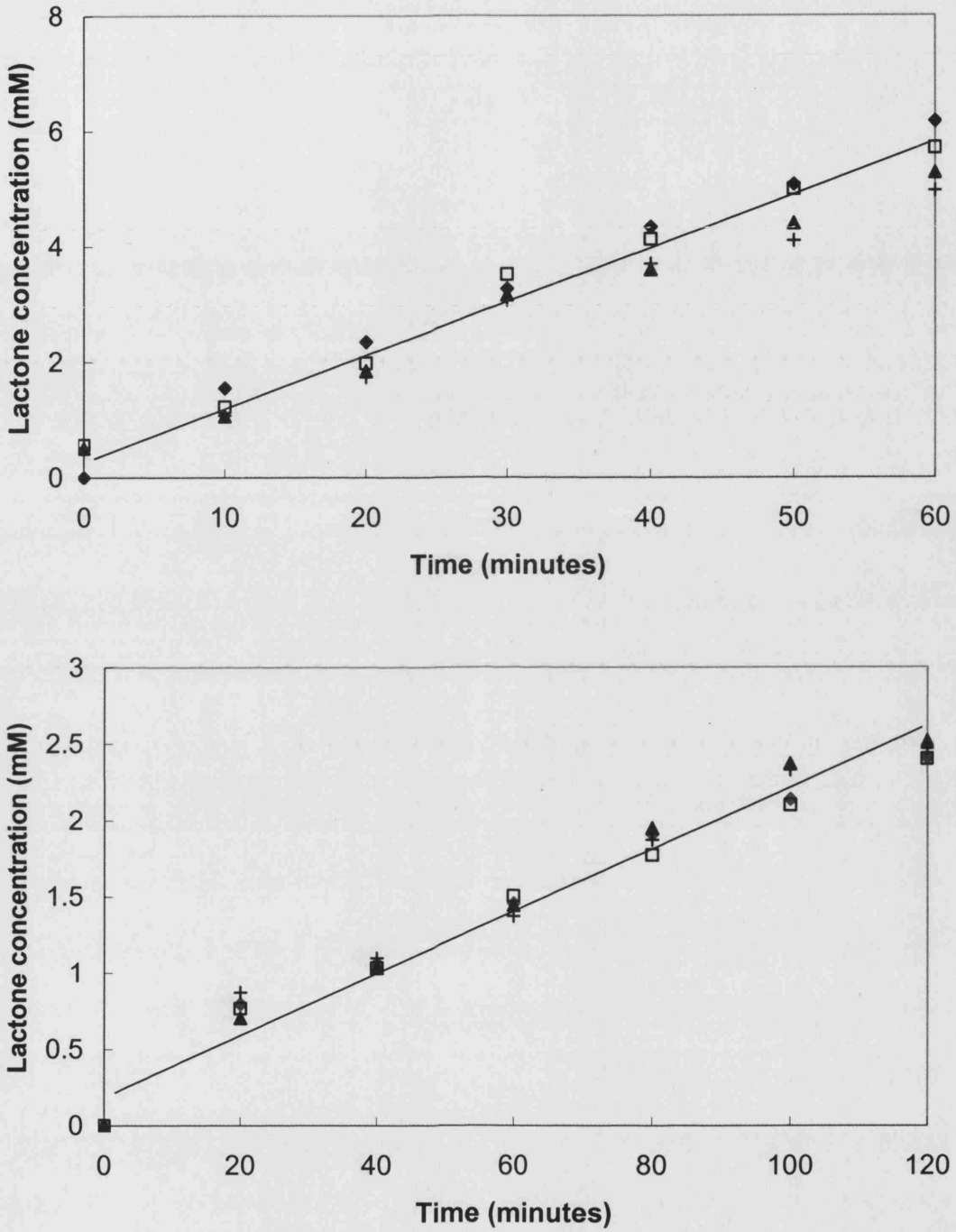


Figure 2.12: The recycleability of free and immobilised whole cells of *E. coli* TOP10 [pQR239], comparison of reaction rates of Bicyclo[3.2.0]hept-2-en-6-one.

◆ 1st run, □ 1st recycle, ▲ 2nd recycle, + 3rd recycle

TOP: Free cells – for recycled runs, cells separated by lab scale centrifuge prior to resuspension in fresh media

BOTTOM: Immobilised whole cells – for recycled runs, beads separated by filter cloth prior to resuspension in fresh media

2.5 Scale-up of whole cell immobilisation

2.5.1 Large-scale cell separation techniques

Production of a high cell density culture of *E. coli* would have reduced the cell-separation processing required. Attempts to use several different defined media (Andersson et al., 1996; Phumathon and Stephens, 1999; Chen et al., 1997; Korz et al., 1995; Chae et al., 1997; Chatterjee and Bhattacharyya, 2001; Reardon, 2000; Walton and Stewart, 2002) which have been used to grow high cell density cultures of *E. coli* elsewhere led to significant reductions in the fermentation growth rate and reduced cell density (data not shown). They were therefore abandoned in favour of the more effective, simpler to prepare and less expensive complex media as previously used on this organism (Doig *et al.*, 2001a). Instead various large-scale separation techniques were employed

As reported in section 1.1.5, immobilised whole cells of *E. coli* have been used industrially for many years, however as yet no information on the feasibility of this has been published. Previous experiments performed at UCL have shown that pilot scale disk stack centrifugation (CSA-1, Westfalia, Milton Keynes, Bucks., UK) and its inherent large shear forces, are catastrophic to many fragile biological materials. Previous (unpublished) attempts to separate *E. coli* cells on a large scale using this disk stack centrifuge (CSA-1) caused a visible deterioration in cell integrity and the enzyme activity of the resultant cell paste was less that 20% of that of the feed.

Thus to enable the large scale separation of cells, a suitable potential cell separation technique was required. There were two potential options, microfiltration or a modern hydro-hermetic disc stack centrifuge.

2.5.1.1 Crossflow microfiltration

Since the 1970's crossflow filtration has emerged as an important tool for cell harvesting and protein purification. It is competitive with centrifugation in

conventional bioprocessing due to its relatively low running and capital costs, modular construction, easy scale up, higher product purity and operation at ambient temperature and in a sterile and contained environment (Shorrock and Bird, 1998; Mulder, 1996). Many centrifuges generate aerosols and heat, have high maintenance costs and are a source of noise pollution (Stratton and Meagher, 1994). Furthermore, the development of genetically modified organisms has led to the need of greater levels of containment (Bailey *et al.*, 1990). Membrane technology has a very low labour requirement (Gatenholm *et al.*, 1998) and offers the versatile tool of diafiltration for buffer exchange and cell washing (Stratton and Meagher, 1994), while in the case of centrifugation cells have to be washed by repeated centrifugation and redilution steps (Tutunjian, 1984). Furthermore, as the membrane physically retains the cells, recovery is essentially 100% with crossflow filtration (Tutunjian, 1984). In a disposables-based plant crossflow filtration offers the additional advantage of potential disposability, which is not an alternative for conventional centrifugation.

2.5.1.2 Disc Stack centrifugation

The application and design of centrifuge separators has been reviewed by Axelsson (1985) and Brunner and Hemfort (1988). There are four main types of centrifuges namely, the tubular bowl, the multi-chamber, the scrolling decanter and the disc stack, which are all used for different process environments. The efficiency of solid and liquid separation is greatly dependent upon particle size, solid-liquid density difference, liquid viscosity, residence time and the relative centrifugation forces (RCF). The disc stack centrifuge is by far the most complex of bowl structures.

Mannweiler and Hoare (1992) reported that the spindle nut positioned at the base of the feed inlet plays a significant role in damaging feed suspensions and this could be the reason for the damage caused to *E. coli* TOP10 [pQR239] on centrifugation with the CSA-1. Neal *et al.* (2003) extended the research to include computational fluid dynamic analysis of the centrifuge during operation. It was established that the maximum shear rate of a pilot-scale disc stack, operating at 7500 rpm, was 1×10^4 s^{-1} and concentrated around the spindle nut. This level of shear was sufficient to reduce

the size of antibody precipitates from an average size of 14.5 μm to 5.3 μm. Byrne *et al.* (2002) and Maybury *et al.* (2000) identified the same region of high shear demonstrating particle breakup upon contact with the spindle nut. Furthermore, Maybury *et al.* (2000) reports that a flow rate below 50 L/h is not sensible because of the temperature build-up in the machine, which can damage the product of interest.

The separator used in the present study is the Westfalia SC-6 disc stack centrifuge installed with a hydro-hermetic feed inlet previously demonstrated to generate gentle conditions during entry of suspensions (Boychyn *et al.* 2000) where the aforementioned majority of cell damage occurs. Other design features include a cooling hood to control overheating of the machine during operation and cleaning-in-place capability for automated cleaning. In some instances however, it is still necessary to dismantle and clean the bowl especially when processing high feed concentrations. Separation begins by feeding material in through the inlet piping positioned at the top of the machine. The material moves down into the distributor and proceeds into the stack of active separation discs where solids and liquids are divided. Cells captured beneath the disks move away from the centre of rotation with a Stokes settling velocity. Stokes settling velocity is very sensitive to the speed of rotation and particle size and it varies as to the second power of both quantities.

Stokes settling velocity is defined by:

$$V = \frac{(\rho_1 - \rho_2)R(\Omega D)^2}{18\mu}$$

where ρ_1 is the solids density, ρ_2 is the liquid density, R is the radius from the axis of rotation, Ω is the angular rotational speed and μ is the liquid viscosity.

The cell paste accumulating at the periphery of the bowl is dispensed at intermittent discharge. The liquid component exits the centrifuge through the supernatant outlet positioned at the top of the centrifuge. The nature of these centrifuges make them ideal for the primary recovery of cells from high density broths (Higgins *et al.*, 1978;

Datar and Rosen, 1987), the removal of cell debris after homogenisation (Mosqueira *et al.*, 1981; Clarkson *et al.*, 1993a), the removal of inclusion bodies (Jin *et al.*, 1994) and for the recovery of precipitates (Bell *et al.*, 1983).

2.5.1.3 Flow cytometry

Simple measurement of cell density by optical density measurement gives no indication of the physiological state of cells. Traditional microbial techniques such as colony counting can show the viability of cells, but the culturing time required provides only historical data which is unsuitable for control purposes. Furthermore, stressed, sub-lethally injured or otherwise viable but non-culturable cells are often undetected. Flow cytometry allows for the measurement of such physiological states in real time and with a high degree of statistical resolution (Hewitt and Nebe-Von-Caron, 2001). A potential concern was found by Hoefel *et al.* (2003) who found that flow cytometry tended to over-estimate the viability of cells by over an order of magnitude compared to plate counts.

Flow cytometry detects cells by their intrinsic light scattering properties in forward angle light scatter (FALS) and right angle (orthogonal) light scatter (RALS), the correlation between cell physiology and light scattering being dependent upon the optical system used (Robertson *et al.*, 1998). Using traditional microbial techniques average values are measured and the physiology of the cell population is often believed to be homogeneous. However flow cytometry has shown that cultures are heterogeneous due to factors such as the cell cycle, imperfect mixing creating microenvironments and genetic differences (Kacmar *et al.*, 2004).

The differing physiology of cells is measured by employing different stains. For exampled bis-(1,3-dibutylbarbituric acid)trimethine oxonol (BOX) is lipophilic and anionic and is useful in assessment of membrane permeabilisation, accumulating intracellularly if the cytoplasmic membrane is depolarized. Propidium iodide (PI) binds to DNA but cannot cross the intact cytoplasmic membrane and is thus a good indicator of cell integrity. By staining and measuring cells in this way the cell

population can be actively sorted by flow cytometry into groups with different physiologies. Because 20-80% of cells with a depolarised membrane (stained by BOX) can be cultured, it is believed that these cells are in a permanent state of flux and can either progress towards permeabilisation and death or revert to being healthy cells (Hewitt and Nebe-Von-Caron, 2001). Generally depolarisation is thought to often result from the lack of an energy source (Reis *et al.*, 2005) and re-polarisation occuring on the introduction of a new energy source.

Flow cytometry has been used for a variety of applications:

- To count the number of cells in a sample (Nebe-Von-Caron *et al.*, 1998).
- To describe the heterogeneity in glucose uptake rates during fermentation (Natarajan and Srienc, 1999).
- To study cell physiology during fermentations (Hewitt and Nebe-Von-Caron, 2001; Lewis *et al.*, 2004; Reis *et al.*, 2005).
- To predict future growth trends from the cell cycle distributions (Abu-Absi and Srienc, 2002).

To date little research has been published on studying cells physiologies during bioconversions, with work by Amanullah *et al.* (2002 and 2003) studying the indene bioconversion being a notable exception.

2.5.1.4 Reproducibility of experiments

A fermentation can yield variations in biomass yield and enzyme activity due to three factors (Schugerl 2005):

1. The quality of the cell culture
2. The fermentation media
3. Process control

Problems with all three were, at one time or another, encountered during this project with a significant proportion of the fermentations being subsequently discarded. To lessen these remaining variations seen with viable fermentations, all free cell and immobilised whole cell reaction runs were run alongside a standard free cell shake

flask experiment using the same batch of cells and the standard bicyclo[3.2.0]hept-2-en-6-one substrate. Similarly to remove variation between isolated enzyme reactions, all reactions were performed alongside a cyclohexanone reaction.

Results were then standardised against these reaction rates to remove any of the variations in enzyme activity resulting from the fermentation or the subsequent storage of cells. Where reactions were to be compared, they were carried out with the same batch of fermentation broth and the same batch of immobilised beads as far as was possible.

Due to the number of different substrates compared, often no errors are represented on graphs, however these are described in the materials and methods section and an indication of errors for the different whole cell reactions is shown graphically in Figure 2.11.

2.5.2 Analysis of scale up alternatives

2.5.2.1 Crossflow microfiltration

The crossflow filtration behaviour was similar to that shown by Tanaka *et al.* (1996), who filtered an *E. coli* broth with a 0.45μm cellulose acetate membrane module; permeation flux followed the cake filtration law at the initial stage of the crossflow filtration, where the cells deposited randomly on the membrane. Then, the specific resistance started to increase due to shear-induced arrangement of the cells. It was found that the average permeation flux was increased considerably with appropriate back washing. Appendix 2 shows the microfiltration performance.

2.5.2.2 SC-6 Centrifugation

Boychyn *et al.* (2001) previously demonstrated how flooding the feed zone prior to feed separation reduces damage to fragile particles. By adopting this method of start-up, negligible *E. coli* cell breakage was visible by the eye at the feed inlet. Cell damage was quantified by flow cytometry in section 2.5.2.4 below.

2.5.2.3 Reaction rate

Figure 2.13 shows the bicyclo[3,2,0]hept-6-en-2-one inhibition profile of *E. coli* TOP10 cells without any cell separation (control), post lab-scale centrifugation, post pilot plant scale disk stack centrifugation and post (cross flow) microfiltration. From the similarity of the profiles it appears that none of the cell separation methods have a negative effect on the enzyme stability and all are potentially suitable.

This result is most surprising for the centrifugation given that previous attempts at scale have produced catastrophic loss of enzyme activity (results not shown) and demonstrates the more modern centrifuge with hydro-hermetic (i.e. with an airtight seal) feed provides a significant process improvement.

2.5.2.4 Flow cytometry

Reaction rate information showed little difference between the alternative cell separation techniques. To assess the stress levels of the different separation techniques on the cell physiology, flow cytometry was employed.

Subsequent to the fermentation (flow cytometry control of 'no unit operation', Figures 2.13 and 2.14), 91% of cells were healthy. The viability of cells has been shown to decline during the stationary growth phase (Kacmar *et al.*, 2004) and so this result was not unexpected. However due to the biocatalytic potential of the cells being greatest during the stationary phase (Doig et al., 2001a), this proportion of non-viable cells must be accepted. It should be noted that the biomass concentrations and specific activities quoted throughout this thesis assumes that all cells have enzyme activity, whereas this result shows this not to be the case. As the same fermentation conditions were used throughout, results have not been altered to reflect this. Furthermore, this result indicates that by improving the fermentation to yield 100% viable cells, as has been shown to be possible elsewhere (Reis *et al.*, 2005), then the specific activity and potentially the volumetric productivity could be increased by 10%.

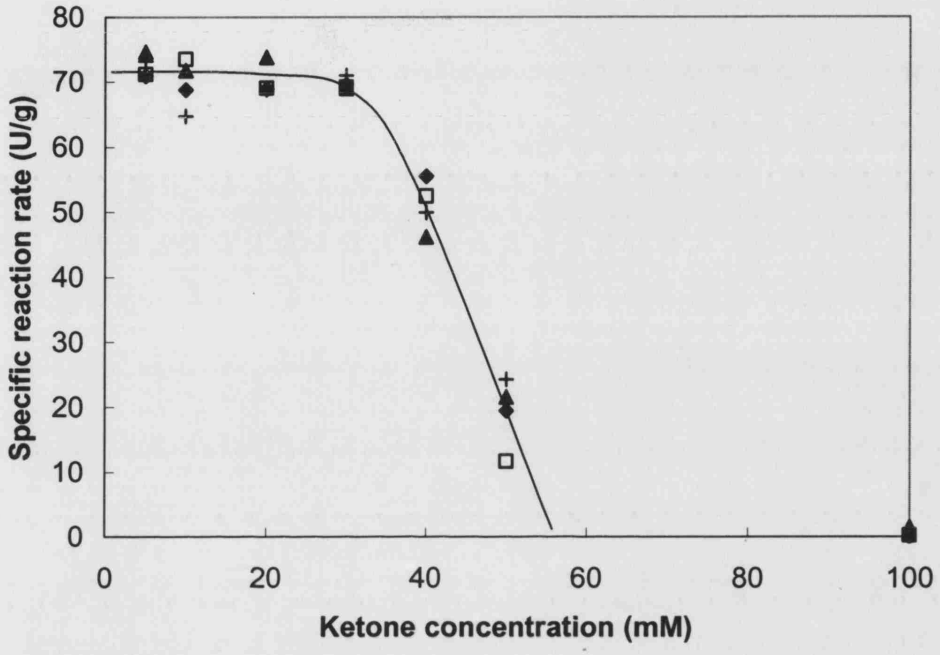


Figure 2.13 – Effect of substrate (bicyclo[3,2,0]hept-6-en-2-one) inhibition on the whole free-cell reaction rates subsequent to the following cell separation techniques:

◆ No unit operation (control)

□ Lab-scale centrifugation

▲ Cross flow microfiltration

\+ Pilot plant scale disc stack centrifugation.

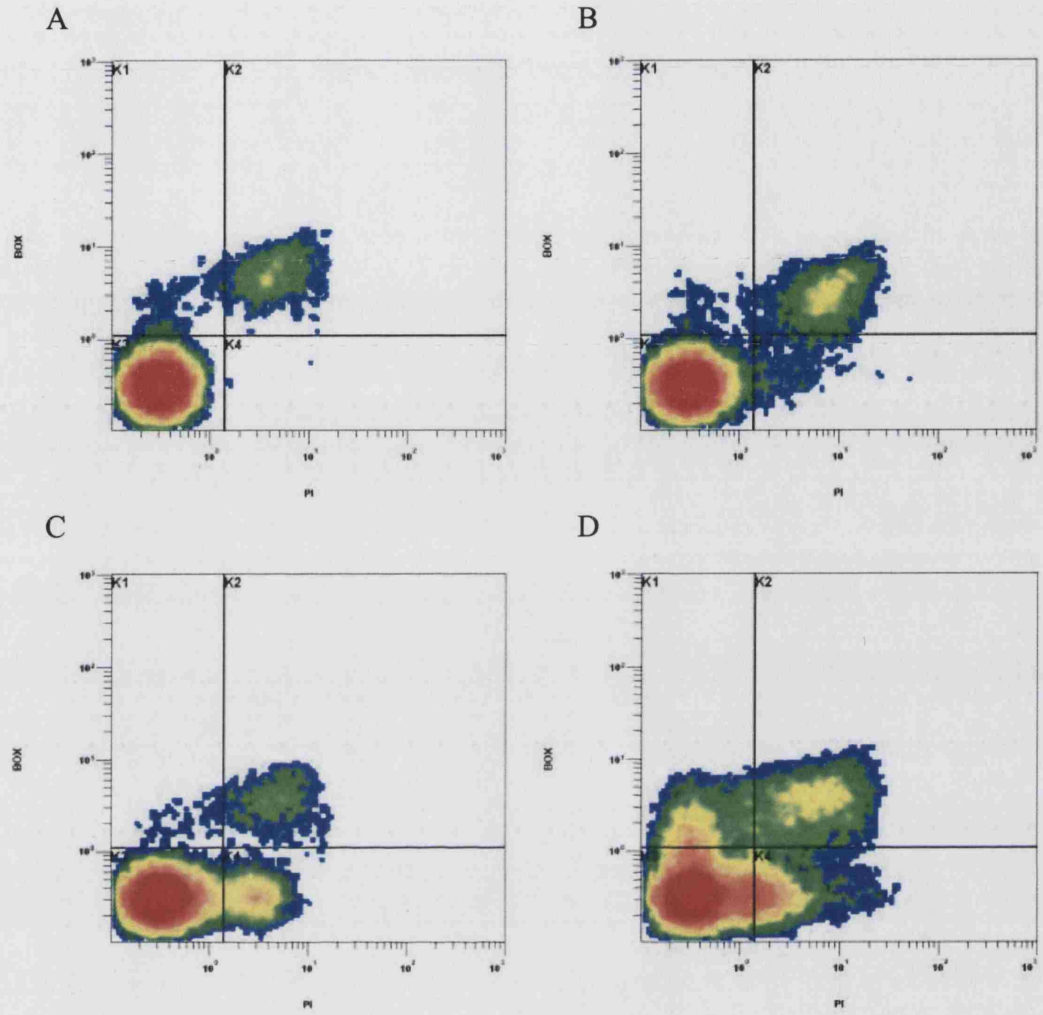


Figure 2.14: Flow cytometry gated diagrams showing the physiological state of the cells post cell separation techniques, but prior to any reaction (percentage healthy cells):

A - No unit operation (91%), B - Lab-scale centrifugation (86%), C - Microfiltration (84%), D - Disc Stack Centrifugation (80%).

Where hotter colours represent a higher number of cells counted in each quadrant, and:

Region K1 (top left quadrant) = depolarised cell membrane; Region K2 (top right quadrant) = depolarised cell membrane, lysed cells; Region K3 (bottom left quadrant) = healthy, i.e. intact cells with a polarised cell membrane; Region K4 (bottom right quadrant) = cell debris. As shown in Figure 2.6.

A potential limitation of flow cytometry is that the size of debris particles cannot be measured, though the sensitivity setting can be adjusted to determine the size of debris fragments that are counted. As a cell can be broken into several particles of debris, each of which are counted, results under-estimate the proportion of healthy cells and are not fully quantitative. In the results presented here the maximum percentage of cell debris fragments counted was 11% with an average of under 3% and therefore the quantitative accuracy is reasonable, though may be underestimating the number of healthy cells by up to 11%.

Flow cytometry pictures (Figure 2.14) show the comparative damage done to *E. coli* cells subsequent to each type of cell separation. The variation in the amount of healthy cells recorded was 82% - 91%. Figure 2.15 shows that when these cells were subsequently used in 1h initial rate reactions (reaction rate data shown in Figure 2.13), that no decline in the cell physiology was apparent at low substrate concentrations.

From the similar specific reaction rates and inhibition profiles seen in Figure 2.13, it appears that the cell separation technique used has no effect. However the flow cytometry diagrams (Figure 2.14) and post reaction healthy cell percentages (Figure 2.15) suggest that the stress caused to the cells follows the following sequence declining harshness scale:
No unit operation > Lab-scale centrifugation > Microfiltration > Disc Stack Centrifugation.
Therefore whilst both microfiltration and disc stack centrifugation could successfully be employed for cell separation prior to scale-up of immobilisation of whole cells, microfiltration appears to be a marginally preferable alternative to centrifugation from these preliminary findings.

A limitation of flow cytometry for use in biocatalysis is that it gives a measure of the viability and physiology of the cells and not of the expressed enzyme activity. It is clear that whilst the higher substrate concentrations seen in Figure 2.13 are inhibitory to the reaction rate, they have less effect on the cell integrity, which shows a more gradual slope. A comparison between the specific reaction rate (Figure 2.13) and the viability of the cells (Figure 2.15) shows that whilst an hour at 100mM is completely

inhibitory to the reaction rate, there is still over 5% of live cells subsequent to all cell separation techniques. These results confirm that ketone is more damaging to the CHMO enzyme and/or cofactor recycling than it is to the cell cytoplasmic membrane.

Amanullah *et al.* (2002) used a measure of the substrate toxicity as being 10% of that of the control. Using this criterion, the bicyclic ketone studied could be classed as toxic to the cells at 40mM for the control (90.2 % of the initial number of healthy cells remained).

From the inhibition profile in Figure 2.13 this appears to be a reasonable indication of when the substrate is inhibitory and thus rather than the process of multiple sampling and GC analysis, flow cytometric analysis appears to be a viable alternative to assessing inhibition.

2.6 Comparison between isolated enzyme, free cell and immobilised whole cell bioconversions

From section 2.5.3.1, oxygen diffusion rates appeared in part to be the rate limiting factor. As the immobilised whole cell reaction appears to have some potential for commercial application as an alternative to free cell reactions, and given the already described importance of inhibition in oxygenase reactions, it seems important to be able to describe further the effects of inhibition between potential biocatalytic systems. To investigate the inhibition seen a range of substrates were selected for study.

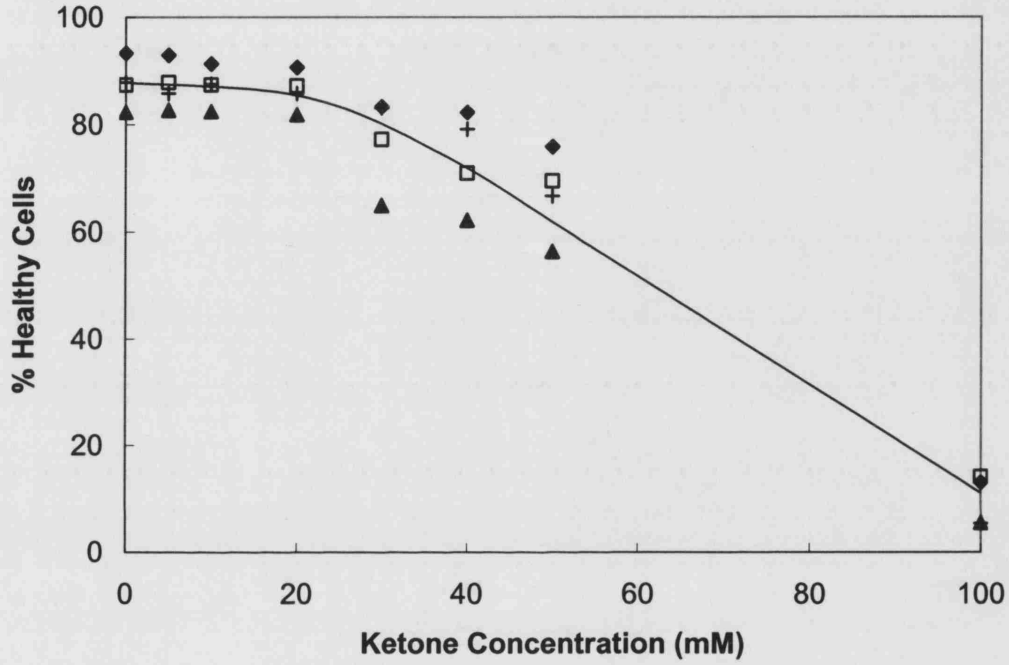


Figure 2.15: Percentage of healthy cells, as recorded by flow cytometry, after 1h reactions with varying substrate concentrations. Data represents the bottom left quadrant (K3) as shown on figure 2.14, where:

◆ No Cell Separation

□ Microfiltration

▲ Disc Stack Centrifugation

+ Lab-scale centrifugation.

Over one hundred substrates have been converted using CHMO biocatalysis (Stewart, 1998). Whilst a number of increasingly complex substrates compatible with CHMO have recently been published, e.g. bicyclic diketones (Ottolina *et al.*, 2005), and benzaldehydes (Moonen *et al.*, 2005), to enable comparison between inhibition results, two different variations around cyclohexanone (the standard substrate of CHMO) were studied:

1. Effects of increasing ring size
2. Effects of increasing chain length

The substrates and their products under CHMO biocatalysis were given in Figures 2.2, 2.3 and 2.4.

2.6.1 Isolated enzyme inhibition

Current research on oxygenase reactions has focused on the use of whole cell biocatalysts rather than the use of isolated enzyme and immobilised enzyme systems due to the complexity and associated expense of cofactor regeneration systems as has already been described in section 1.1.6. To this end the inhibition observed with such systems is perhaps of less importance than that seen in free and immobilised whole cell systems, however, to be able to assess the effectiveness of the free and immobilised whole cell systems, knowledge of the isolated enzyme reaction rate and an idea of the inhibitory profile is required.

Effect of ring size on substrate inhibition – The general inhibitory trend is similar for all ring sizes as shown in Figure 2.16. Generally by increasing ring size the enzyme activity appears to also increase, this holding true at all ketone concentrations. This is a somewhat unexpected result given that the enzyme was named after cyclohexanone. From 2mM to 20mM the enzyme activity appears to drop gradually by 25%, from then on up to 50mM the inhibition appears to be constant. At 50mM the substrate still appears not to be fully inhibitory. This was an unexpected result, with the cell membrane expected to offer protection to the CHMO activity, and suggests that it is either the cofactor recycling within the cell that is damaged at high ketone concentration or that inhibition is a factor of time as well as substrate concentration.

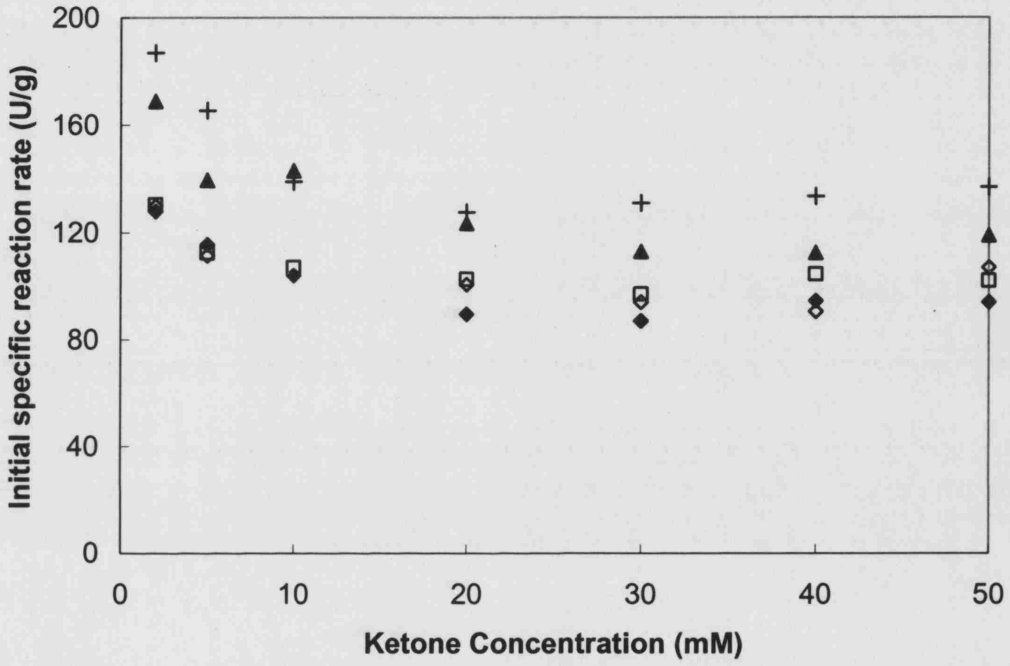


Figure 2.16: Effect of ring size on isolated enzyme activity. ◆ cyclobutanone, □ cyclopentanone, ▲ cyclohexanone, + cycloheptanone, ◇ bicyclo[3.2.0]hept-2-en-6-one.

The reduced apparent inhibition at the fast reaction times (two minutes) was independently observed when at high substrate concentrations the free cell biocatalyst showed limited reaction potential over the first ten minutes of reaction, after which little/no conversion was seen.

The structural effect of adding an additional ring to cyclobutanone to make the bicyclic ketone appears to have very little effect on the enzyme activity seen. This may suggest that the apparent isolated enzyme activity is a function of the shape of the substrate molecule.

Effect of chain length on substrate inhibition – As shown in Figure 2.17, the enzyme activity of 4-methyl cyclohexanone and, to a lesser extent 4-ethyl cyclohexanone, is generally greater than that seen for cyclohexanone at all substrate concentrations. The substrates of increasing chain length were specifically selected so that the additional chain is opposite the reactive oxygen bond. As there is a variation seen with the different substrates it appears that the enzyme activity is not simply a function of the molecule's shape, but other factors potentially, such as the aqueous solubility or diffusivity of the substrate.

The decrease in enzyme activity is greatest between low level concentrations of ketone, with a gradual decrease up to 30mM, and then constant activity from 30 to 50mM. The general inhibitory profile for all substrates is similar to that shown with increasing ring sizes, which suggests that this pattern could be demonstrative of that seen with any substrate.

Effect of number of rings on product inhibition – Figure 2.18 shows the product inhibition profile for cyclohexanone and bicyclo[3.2.0]hept-2-en-6-one. Both products appear to have little effect on the enzyme activity over the concentration range studied. As the product is known to be less inhibitory than the substrate on free cell reactions, given that the substrates are showing little inhibition for isolated enzymes, then a similar result would be expected from the products also.

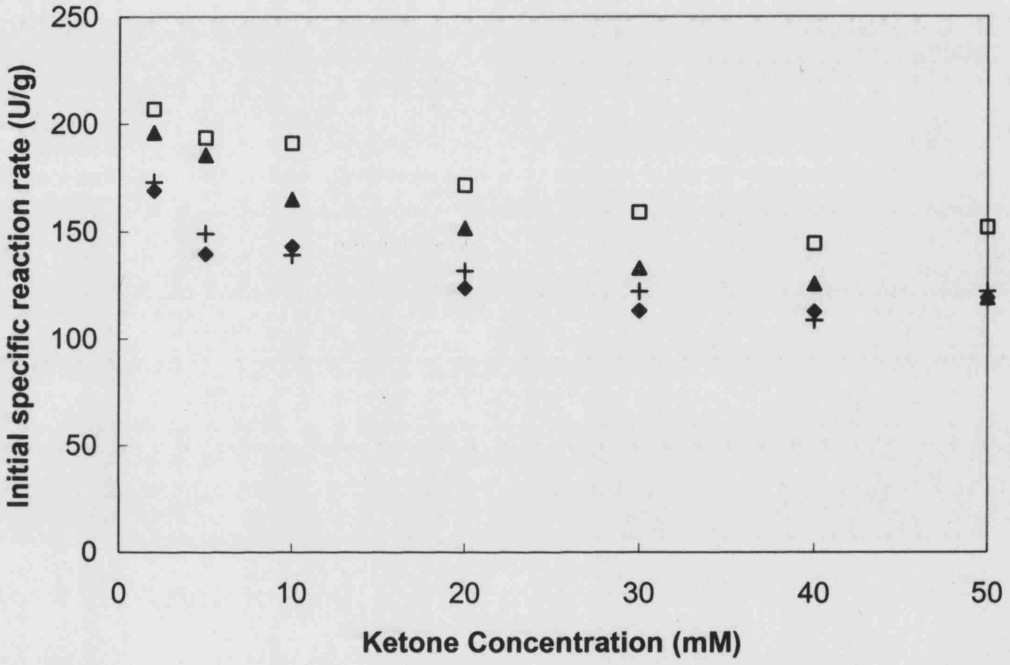


Figure 2.17: Effect of chain length on isolated enzyme activity. ◆cyclohexanone, □ 4-methyl cyclohexanone,▲ 4-ethyl cyclohexanone,+ 4-propyl cycloheptanone

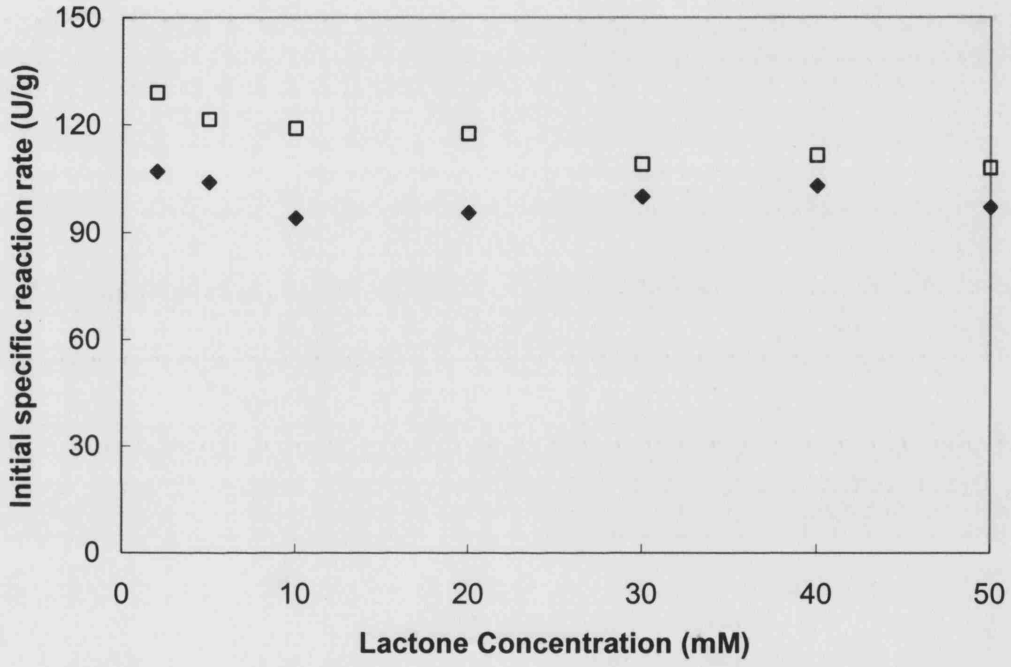


Figure 2.18: Effect ring size and product inhibition on isolated enzyme activity on 5mM ketone. Where ◆ (-) 1(R), 5(S) 2-oxabicyclo[3.3.0]oct-6-en-3-one, □ e-caprolactone.

2.6.2 Free cell inhibition

The most noticeable difference between the reaction rates in free cells (Figures 2.19 & 2.20) and the enzyme activity seen for different ring sizes (Figures 2.16 & 2.17) is the increased spread in the results. As free cell reaction rates are known to be mass transfer limited, the differences seen are likely to be a result of the physical properties of the substrates.

Effect of ring size on substrate inhibition – In free whole cells the reaction rate is roughly two-fold higher in cyclohexanone than in the other ring sizes (Figure 2.19), with that of bicyclo[3,2,0]hept-6-en-2-one being higher still. Interestingly the approximate level at which substrates become inhibitory (Table 2.5) increases for reducing ring size for the range of compounds studied. There appears to be little link between the reaction rate seen in whole cells and the levels at which they become inhibitory, i.e. substrates of smaller ring sizes appear to have a lower inhibitory effect than those of the larger ring sizes (cyclohexanone and cycloheptanone) which yield higher rates of reaction. This could further indicate that the reaction rate is substrate mass transfer limited rather than being dependent upon the intrinsic reaction rate as postulated by Doig *et al.* (2001).

Substrate	Apparent inhibitory concentration (mM)
cyclobutanone	>144
cyclopentanone	>67
cyclohexanone	55
cycloheptanone	20
bicyclo[3.2.0]hept-2-en-6-one.	23

Table 2.5: Approximate apparent inhibitory levels of substrates of increasing ring size. Values are taken from dashed lines drawn on Figure 2.19.

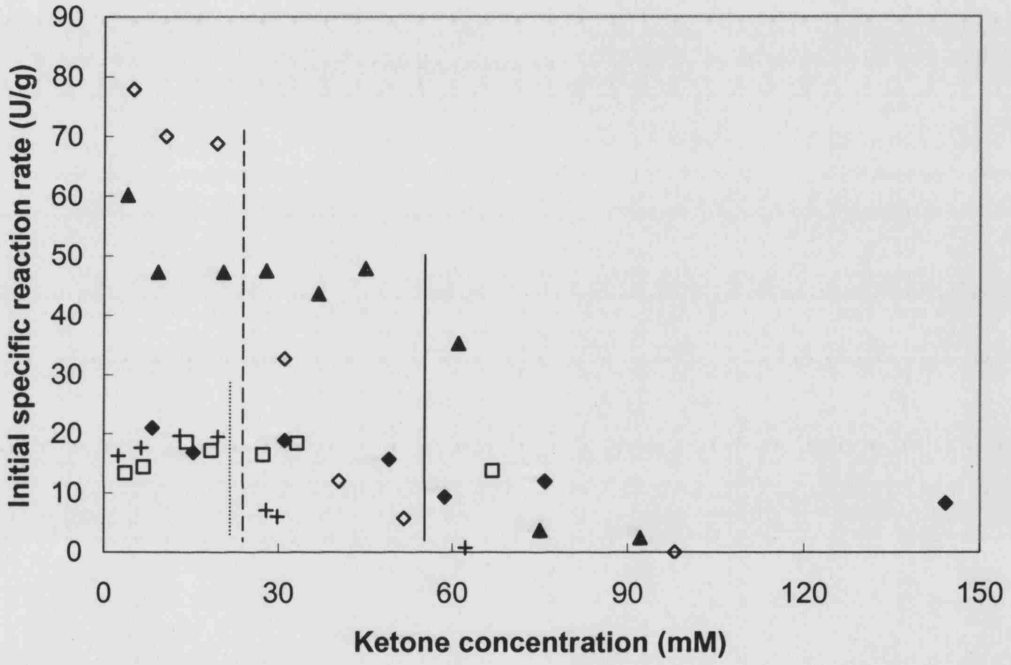


Figure 2.19: Effect of ring size on free cell reaction rate: ◆ cyclobutanone, □ cyclopentanone, ▲ cyclohexanone, + cycloheptanone, ◇ bicyclo[3.2.0]hept-2-en-6-one.

Lines are marked where inhibition appears to become significant (defined as <75% of 5mm initial specific reaction rate):

—— cyclohexanone, ····· cycloheptanone, – – – bicyclo[3.2.0]hept-2-en-6-one.

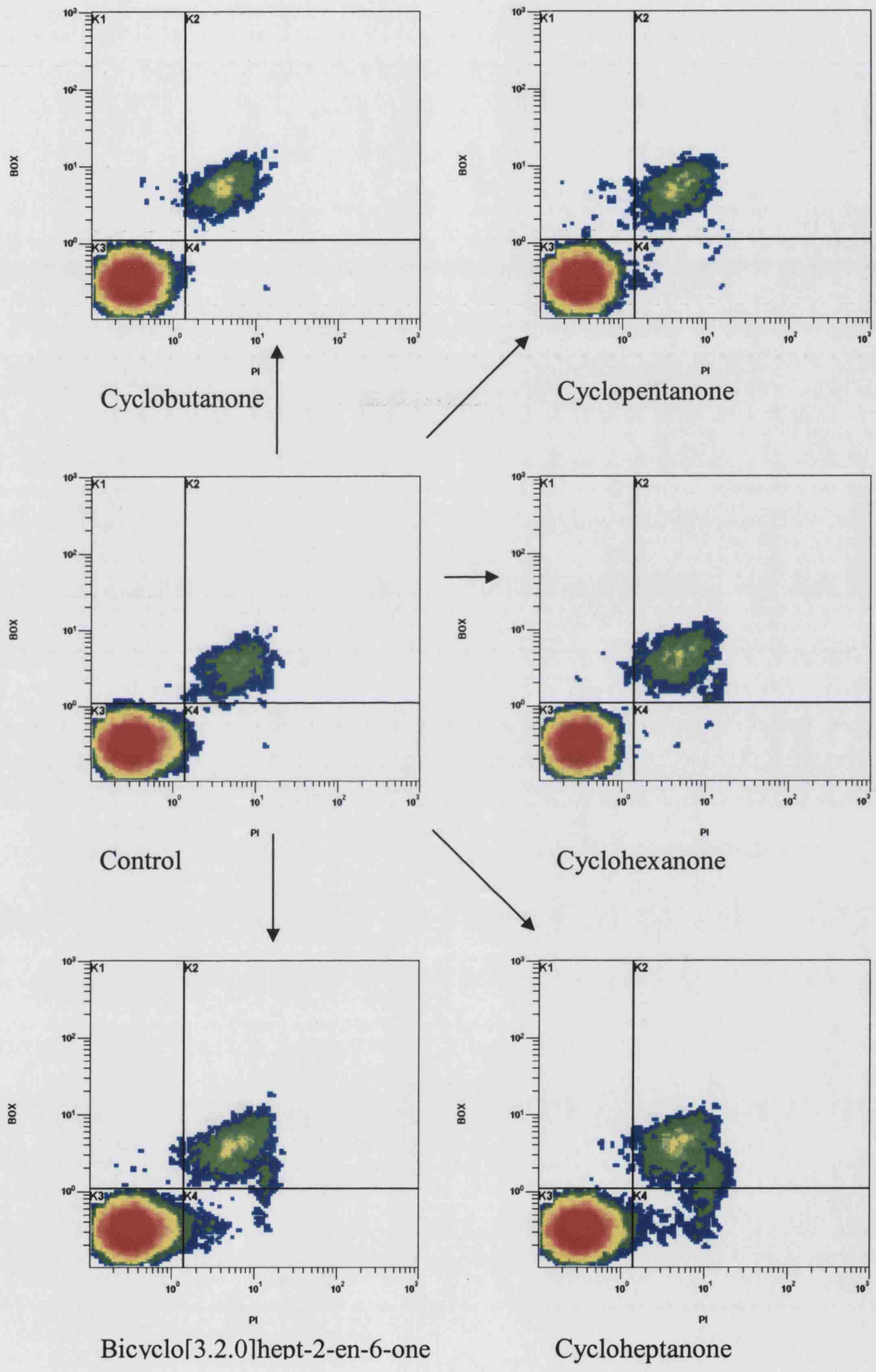


Figure 2.20: Effect of substrate (10mM) ring size on integrity of cells, as measured by flow cytometry.

The effect of increasing bicyclo[3,2,0]hept-6-en-2-one concentration on cell physiology, as measured by flow cytometry, was shown in Figure 2.15. Due to the sample preparation and analysis times it was not feasible to repeat this for all the substrates studied. Whilst little inhibition was seen with substrates at 10mM concentrations, due to the increased sensitivity of flow cytometry this concentration was selected to enable assessment of the potential damage to cells such apparently low concentrations. These cytometry pictures are shown in Figure 2.20.

From these results, cyclobutanone has the least effect on the percentage of healthy cells (92%), with cyclopentanone and cyclohexanone giving similar results (91%) and cycloheptanone being marginally more damaging to the cells (87%). Bicyclo[3.2.0]hept-2-en-6-one, being slightly less inhibitory to the CHMO activity, is also slightly less toxic to the cells (89%) than cycloheptanone. It appears that the damaging effect of all the substrates on the cell cytoplasmic membrane follows the same pattern as its inhibitory effect on CHMO. However cyclopentanone is slightly less inhibitory than cyclohexanone, whilst its effect on the cell physiology is negligible. Therefore, whilst flow cytometry provides a good indication of the toxicity and thus likely inhibitory effect of the substrate on the cell membrane, it cannot be exclusively relied upon as an alternative to GC monitoring.

Effect of chain length on substrate inhibition – Whilst the reaction rate at lower substrate concentrations of cyclohexanone and 4-methyl cyclohexanone is similar, further increases in chain length result in significant (approximately 50%) reductions in reaction rate (Figure 2.21). Similarly the level at which the substrate becomes inhibitory (Table 2.6) appears to decrease by around 50% for increasing chain length. The demonstrated inhibitory level of each substrate is also comparable to apparent toxicity of each substrate as shown be flow cytometry (Figure 2.22), where generally increasing chain lengths were more damaging to the cells physiology. Cyclohexanone was clearly the least harmful to the cells (91% healthy) and the increasing chain length caused decreasing levels of healthy cells, 4-methyl cyclohexanone (90%), 4-ethyl cyclohexanone (82%), 4-propyl cyclohexanone (15%).

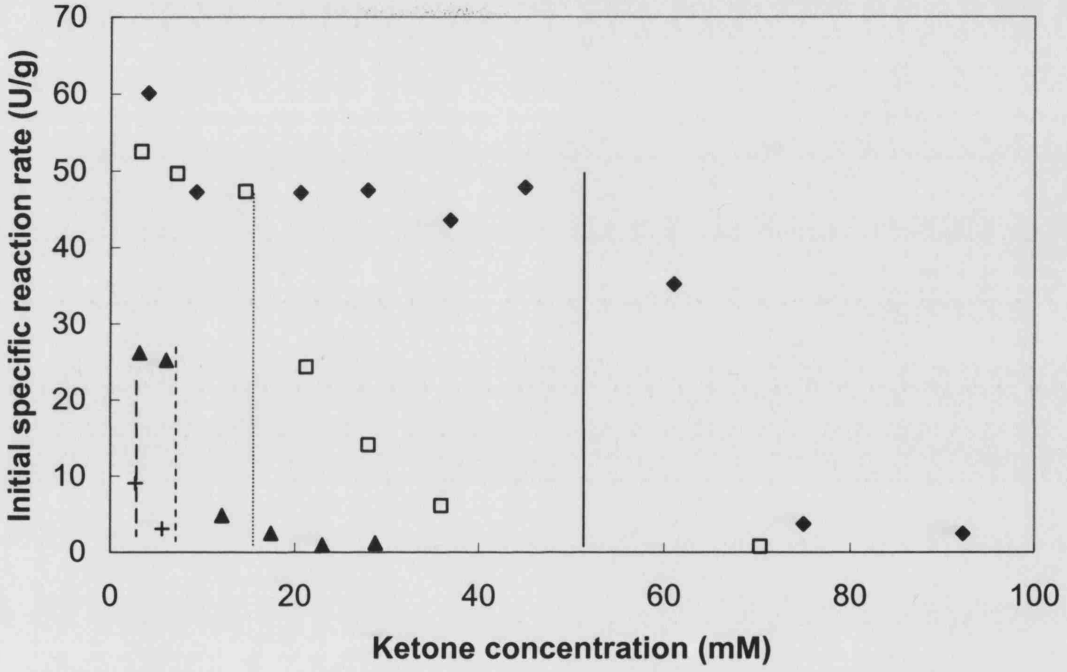


Figure 2.21: Effect of chain length on free cell reaction rate. ◆ cyclohexanone, □ 4-methyl cyclohexanone, ▲ 4-ethyl cyclohexanone, + 4-propyl cycloheptanone

Lines are marked where inhibition appears to become significant:

—— cyclohexanone, ... 4-methyl cyclohexanone, ---- 4-ethyl cyclohexanone, – – – 4-propyl cycloheptanone

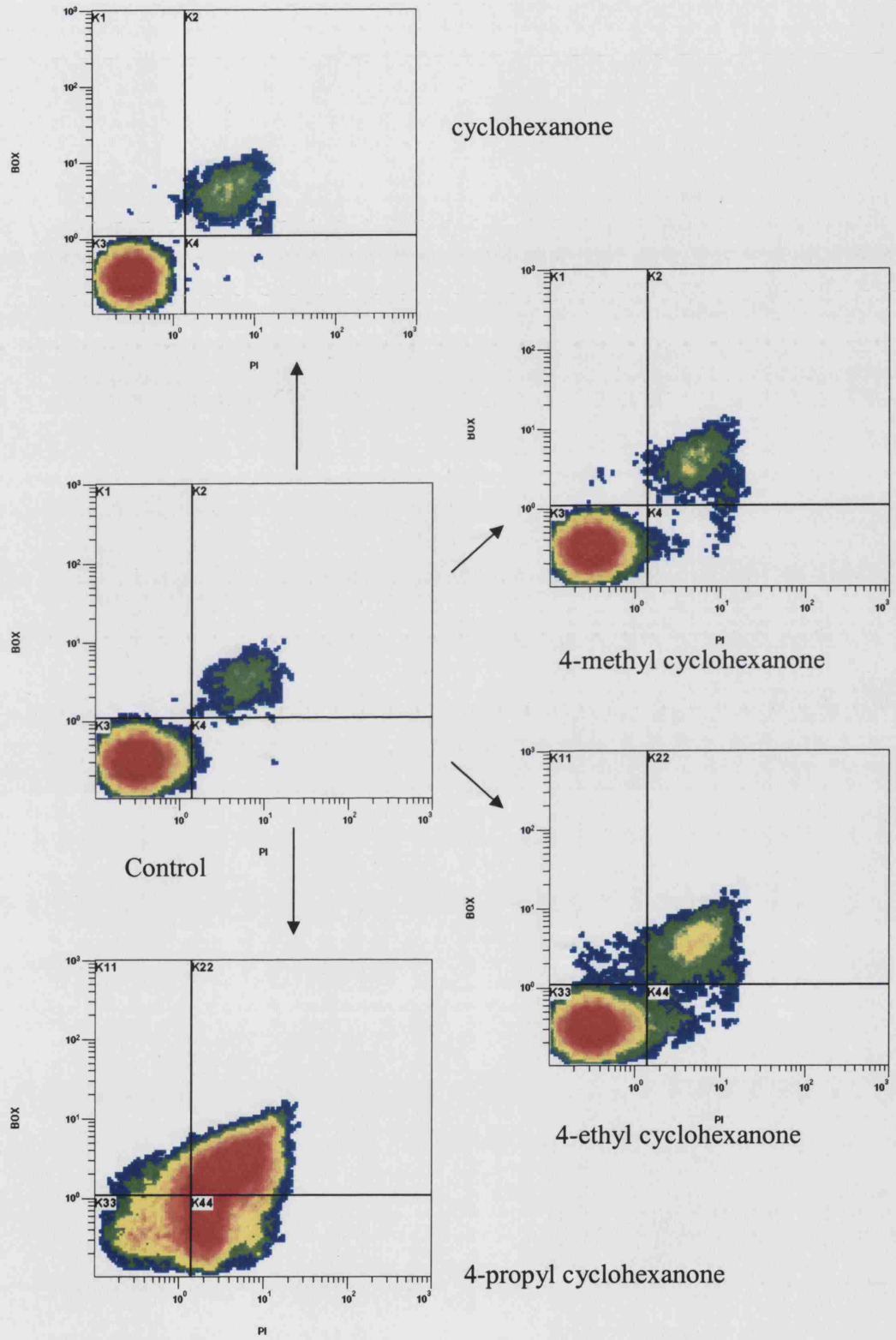


Figure 2.22: Effect of chain length on integrity of cells, as measured by flow cytometry.

The flow cytometry result for post 4-propyl cyclohexanone reaction is similar to that shown in the partially stressed cells post centrifugation (Figure 2.14), caution must be exercised over the quantitative percentage of healthy cells shown due to the inability to effectively gate the differing cell physiologies. It should be noted that of all the substrates that were tested, only 4-propyl cyclohexanone was considered significantly volatile, with around 50% of the substrate evaporating over a one hour test of volatility (Appendix VI, Figure AVI.1). If substrate volatility is also indicative of its cell toxicity, it appears reasonable that this substrate would become inhibitory at a lower concentration and also show the least reactivity. From this result it could be suggested that 4-propyl cyclohexanone is partially permeabilising the cell membrane and the reduced protection to the CHMO could explain why there was no conversion seen at substrate concentrations above 5mM for this substrate.

Substrate	Apparent inhibitory concentration (mM)
cyclohexanone	55
4-methyl cyclohexanone	16
4-ethyl cyclohexanone	8
4-propyl cyclohexanone	3

Table 2.6: Approximate apparent inhibitory levels of substrates of increasing chain length. Values are taken from dashed lines drawn on Figure 2.20.

Effect of number of rings on product inhibition of ketone consumption – From Figure 2.23, the effect of product inhibition is more linear than that of substrate inhibition with increasing product concentration. Given the close relationship between the substrates and products, a correlation between the substrate and product inhibition levels was expected for the two different substrates. However, the approximate significant inhibitory level of the bicyclic ketone (Table 2.5) is approximately half of that of the corresponding lactone (Table 2.7), whereas cyclohexanone (Table 2.6) becomes significantly inhibitory at a level around 50% higher than its product e-caprolactone (Table 2.7). Whilst this is an interesting result, the lack of other

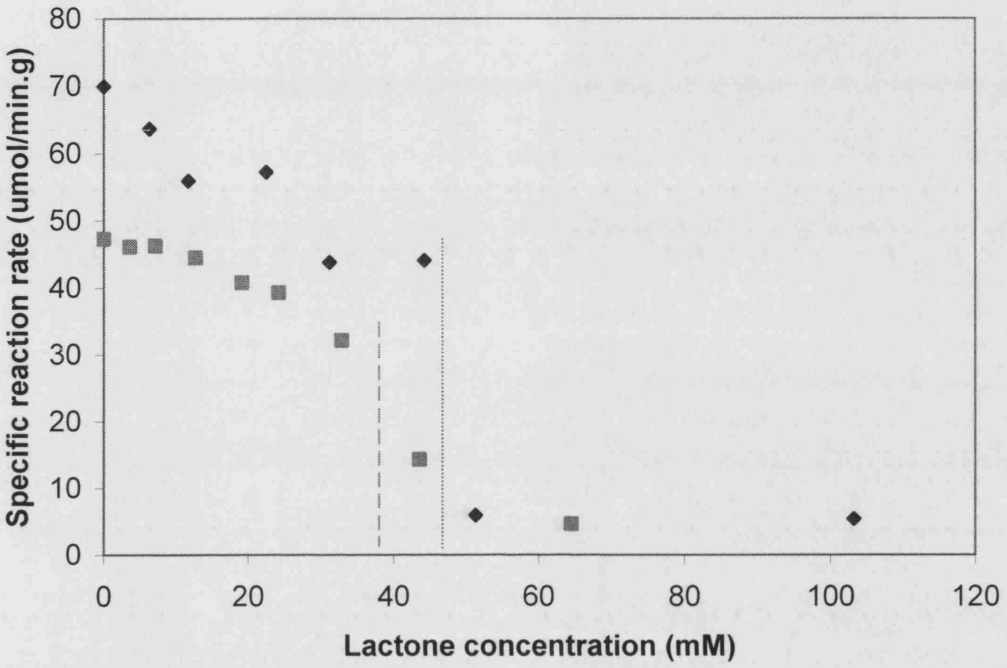


Figure 2.23: Effect of number of rings on product inhibition of free cells.

Where:

◆ (-) 1(R), 5(S) 2-oxabicyclo[3.3.0]oct-6-en-3-one, ■ e-caprolactone.

Lines are marked where inhibition appears to become significant: ····· (-) 1(R), 5(S) 2-oxabicyclo[3.3.0]oct-6-en-3-one – – – e-caprolactone

commercially available products and the difficulty in either producing these products chemically (section 2.2.6.2.1) or purifying them from biocatalytic routes, made further investigation not possible in this study.

Product	Apparent inhibitory concentration (mM)
(1R,5S) 2-oxabicyclo[3.3.0]oct-6-en-3-one	46
e-caprolactone	38

Table 2.7: Approximate apparent inhibitory levels of the two commercially available products. Values are taken from dashed lines drawn on Figure 2.21.

2.6.3 Immobilised whole cell inhibition

Chung *et al.* (2003) compared phenol degradation inhibition and reaction rate for *Pseudomonas putida* both free and cells immobilised in calcium alginate. They found that the immobilised cells could tolerate higher inhibitory substrate concentrations when immobilised (1g/L compared to 0.6g/L for free cells). To assess whether immobilisation increases the substrate tolerance with CHMO, reactions comparable to those seen with free cells were performed.

Effect of ring size on substrate inhibition – With immobilised whole cells (3mm bead size) the reaction rate (Figure 2.24) appears much more sensitive to increasing ketone concentration for the more reactive substrates of bicyclo[3.2.0]hept-2-en-6-one and cyclohexanone than was seen with free cells (Figure 2.19). For example the reduction in reaction rate when the ketone concentration increases from 5mM to 10mM for cyclohexanone and bicyclo[3,2,0]hept-6-en-2-one is 79% and 90% of the reaction rate at 5mM respectively for free cells and 43% and 51% respectively for immobilised whole cells. This suggests that rather than the immobilisation increasing the resistance of the enzyme to levels of substrate, it actually reduces it. A possible explanation for this is that the partial oxygen mass transfer limitation (section 2.5.3.1) on the reaction makes the cells less able to maintain complete cell function and thus at high substrate concentrations and the cell struggles to survive.

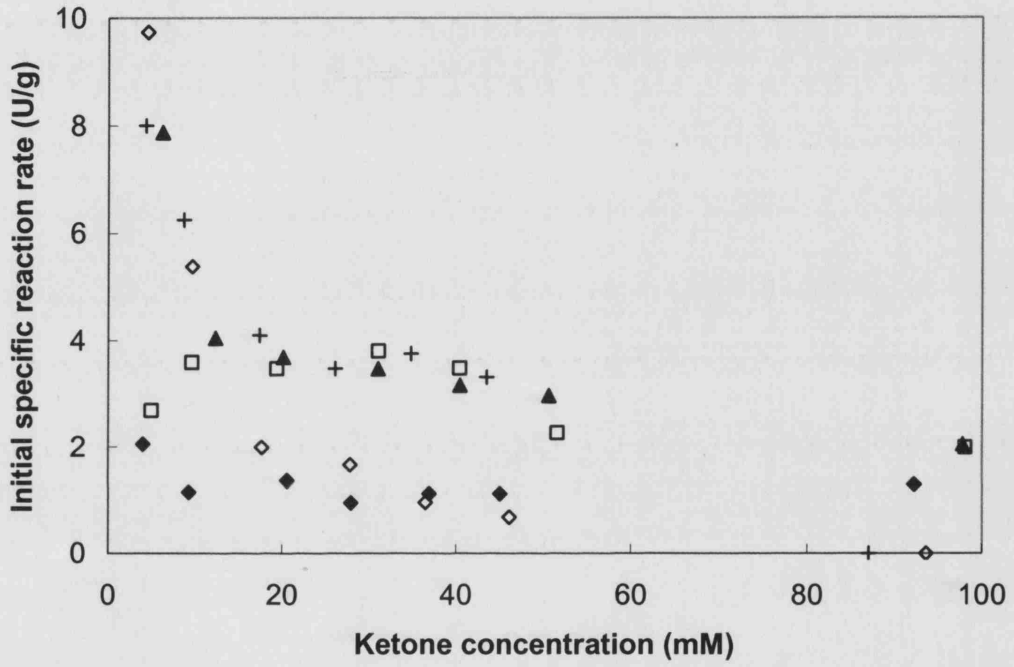


Figure 2.24: Effect of ring size on immobilised whole cell reaction rate. Where:
◆ cyclobutanone, □ cyclopentanone, ▲ cyclohexanone, + cycloheptanone,
◇ bicyclo[3.2.0]hept-2-en-6-one

Interestingly the increased sensitivity of reaction rate to substrate concentration that was apparent with the faster reacting substrate (noted above) was not apparent with the slower reacting substrates. Taking the assumption that the molar requirement for oxygen is similar for all reactions, the reduced oxygen requirement for slower reactions is likely to increase the availability of oxygen required for respiration and metabolism. It appears reasonable to suggest that this is likely to improve both the viability and enzyme activity of the cells and thus reduce the apparent effect of inhibition for these substrates.

A special noteworthy case is that of cycloheptanone, which yielded slow reaction rates at low concentrations in free cells (around 25% of that of cyclohexanone) and a comparably fast reaction rate (similar to cyclohexanone) in the immobilised whole cells and with the isolated enzyme. This result will be discussed further in section 2.7.4.

Effect of chain length on substrate inhibition – As was seen with substrates of increasing ring size, the immobilised whole cells reaction rate (Figure 2.25) is again more sensitive to increasing ketone concentration that was seen with the free cells (Figure 2.21).

For 4-ethyl cyclohexanone there was no apparent rate of reaction above concentrations of 8mM and for 4-propyl cyclohexanone there was no reaction seen at all. This is further evidence that the immobilisation of whole cells has in fact made the cells more prone to inhibition than free cells, rather than leading to an increase in stability. This appears to be conclusive evidence that in immobilised systems the reaction rate is predominantly oxygen mass transfer limited rather than substrate mass transfer limited, and furthermore suggests that lack of available oxygen seen in the immobilised systems is more damaging to the CHMO activity than excess oxygen was proven to be by Doig *et al.* (2003).

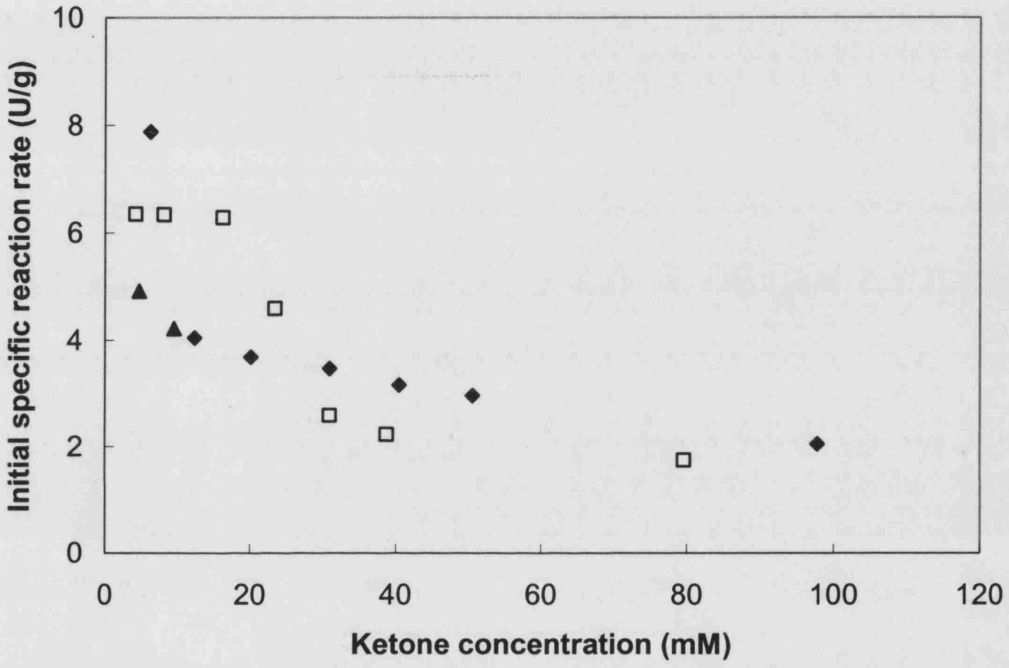


Figure 2.25: Effect of chain length on immobilised whole cell reaction rate.
◆ cyclohexanone, □ 4-methyl cyclohexanone, ▲ 4-ethyl cyclohexanone.

Effect of number of rings on product inhibition of ketone consumption – Comparing Figures 2.23 and 2.26 the inhibition profiles appear to be very similar for both products. The only visible difference was that for immobilised whole cells the effect of product concentration is gradual and almost linear, in comparison, in free cell reactions there was a definite level at which an approximately inhibitory concentration could be identified. Also worthy of note is that at very high product concentrations (100mM) a larger percentage of the initial reaction rate remained with immobilised cells. This is of a similar actual reaction rate as that observed with free cells and perhaps is more indicative of the time-dependent nature of inhibition as was identified in section 2.4.2.

2.6.4 Comparison between isolated enzyme, free cell and immobilised whole cell systems

To enable a comparison between the different CHMO catalysed reactions, the catalyst effectiveness factor has been calculated as defined by Liu *et al.* (2005):

$$\eta = \frac{\text{Rate with diffusion resistance}}{\text{Rate without diffusion resistance}} = \frac{\text{Observed rate}}{\text{Intrinsic rate}}$$

In other words it is the reaction rate evaluated at the outer surface compared to that internally. Thus the effectiveness of the immobilised whole cells compared with free cells can be quantified using an analogous equation

$$\eta = \frac{\text{Rate in immobilised whole cells}}{\text{Rate in free whole cells}}$$

Where η is a measure of the comparative mass transfer limitation.

As the effect of different substrate concentrations on the inhibition profiles have already been discussed above, only the effect of substrate concentrations of 5mM will be used here for a comparison between CHMO systems.

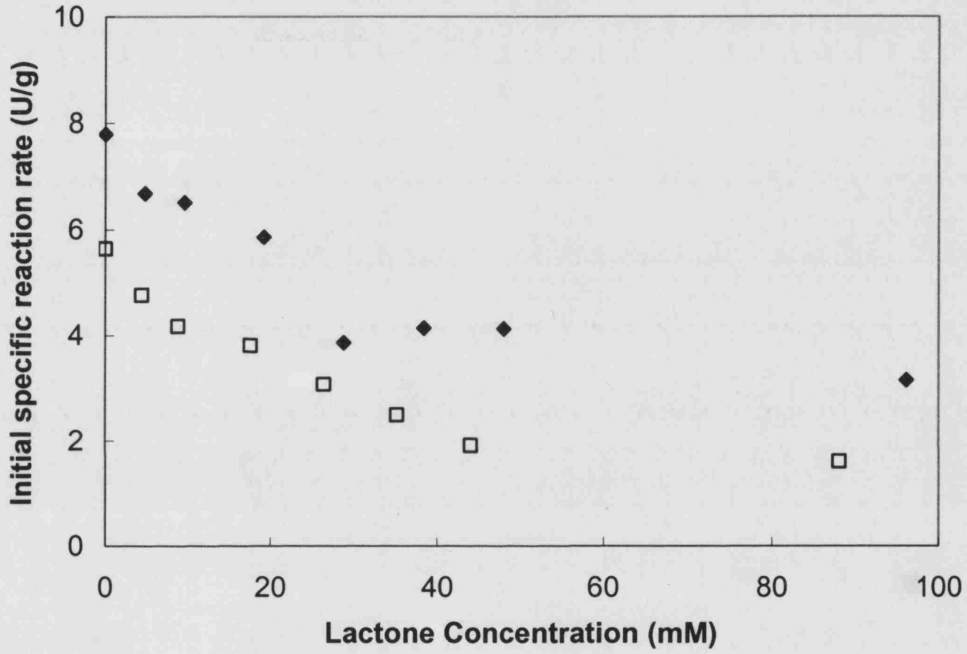


Figure 2.26: Effect ring size on product inhibition of immobilised whole cell reaction rate.

◆ (-) 1(R), 5(S) 2-oxabicyclo[3.3.0]oct-6-en-3-one, □ e-caprolactone.

The catalyst effectiveness factors for both free cells compared to isolated enzyme and for immobilised whole cells in comparison to free cells are shown in Figures 2.28 & 2.30. Different profiles between different substrates are apparent for the effectiveness factors of whole cells and immobilised whole cells. As the rate of reaction with free cells must be substrate mass transfer limited (oxygen being available in excess), the differing profiles either confirms the predominantly oxygen diffusion limited reaction rate apparent with immobilised whole cells or, potentially suggests that the mass transfer of substrates into the free whole cells is not solely based on passive diffusion, but also contains active transport mechanisms.

Effect of ring size – Actual specific reaction rates for isolated enzyme, free whole cells and immobilised whole cells are compared in Figure 2.27. The predominant result from this data is the unexpectedly fast specific reaction rate apparent with bicyloheptanone in free cells in comparison to the reaction rate with the isolated enzyme.

Given the reaction rates for each substrate are comparable to cyclohexanone in the isolated enzyme reactions (true enzyme reaction rate limited), the mass transfer rate of substrate into the cell must be considerably slower for different ring sizes.

It appears that substrates with faster specific reaction rates also give higher product yields (Table 2.8). As the volatility of the substrates has been assessed as being low (except for 4-propyl cyclohexanone), and comparably the substrates tend to be more volatile than the products (e.g. the bicyclic ketone is a liquid whilst its corresponding lactone is a solid at room temperature), the reason for the difference in yields is unclear. Possible alternative methods of loss of substrate could be via accumulation in the cells or through molecular breakdown.

The purity of the product (*ee*) was high in all cases and was greater than the limits of detection on the GC system for all substrates except bicyclo[3.2.0]hept-2-en-6-one which merits special discussion.

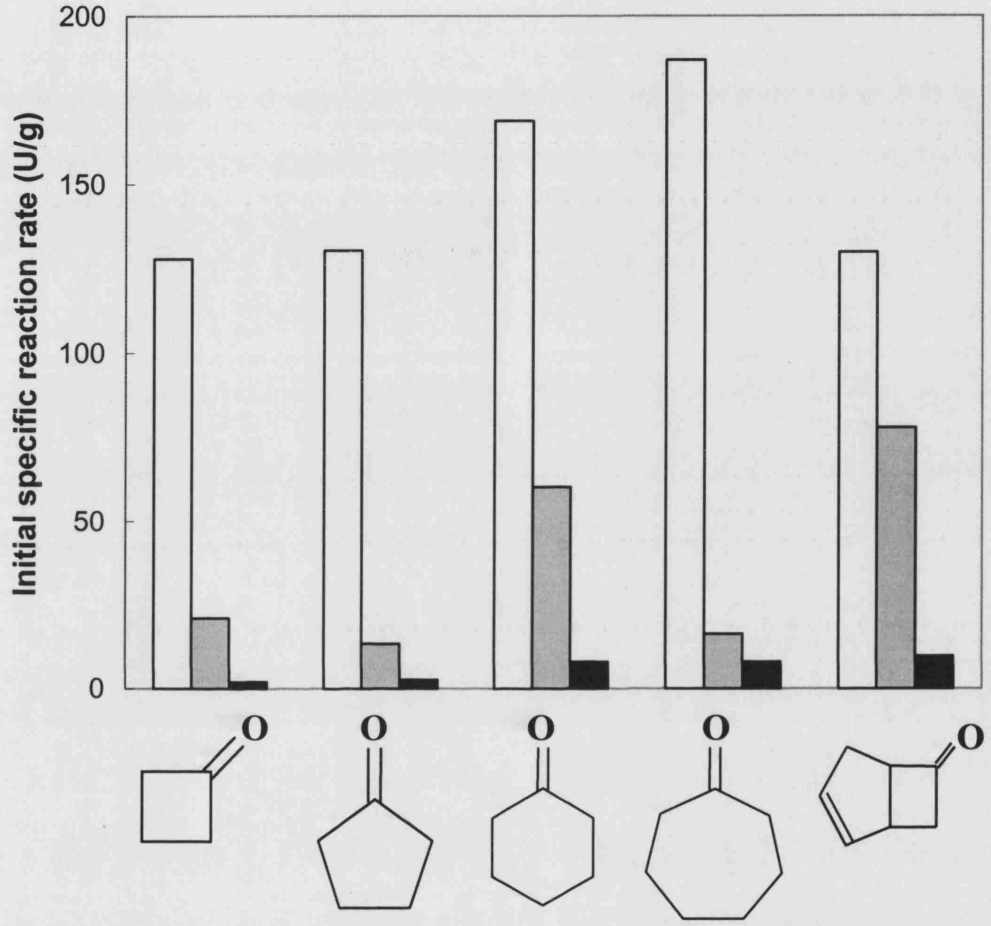


Figure 2.27: Effects of ring size on a comparison of the initial specific activity of isolated enzyme, whole cells and immobilised whole cells of *E. coli* TOP10 [pQR239] for the oxidation of 5mM of (left to right) cyclobutanone, cyclopentanone, cyclohexanone, cycloheptanone and bicyclo[3.2.0]hept-2-en-6-one.

Where:

□ Isolated enzyme, ▒ Free cells, ■ Immobilised whole cells

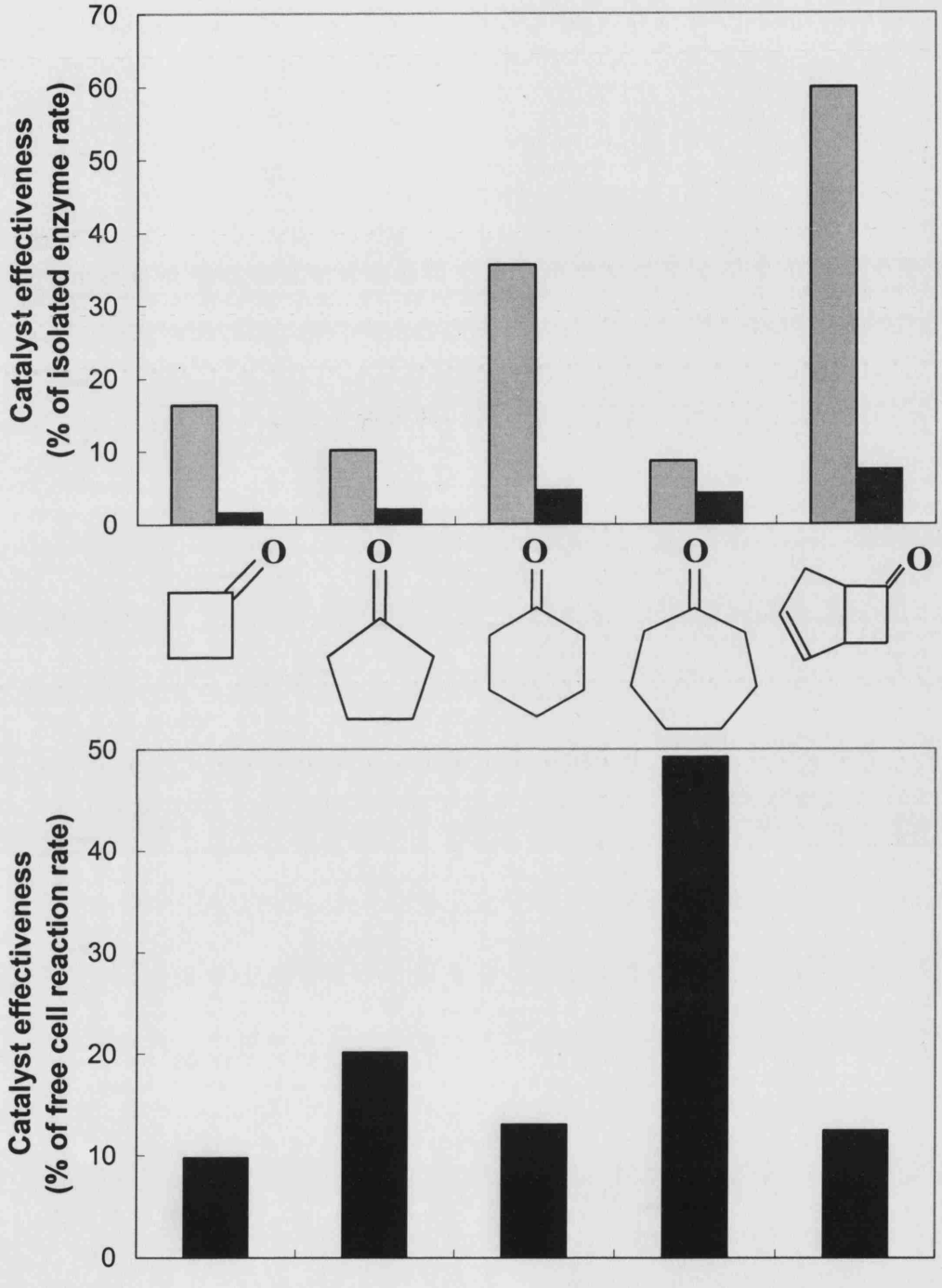


Figure 2.28: Effects of ring size on a comparison of the initial specific activity of whole cells and immobilised whole cells of E. coli TOP10 [pQR239] for the oxidation of (left to right) cyclobutanone, cyclopentanone, cyclohexanone, cycloheptanone and bicyclo[3.2.0]hept-2-en-6-one.
Where:
Free cells, Immobilised whole cells
Top: Free and immobilised whole cell reaction rates as a percentage of isolated enzyme reaction rate. Bottom: Immobilised whole cell reaction rate as a percentage of free cell reaction rate.

Substrate	Free cell yield	Free cell *ee*	Isolated enzyme, free cell and Immobilised whole cell specific reaction rate (U/g)
cyclobutanone	52%	>95%	128 21.0 2.1
cyclopentanone	64%	>95%	130.5 13.3 2.7
cyclohexanone	80%	>98%	169 60.2 7.9
Cycloheptanone	72%	>95%	187 16.3 8.0
bicyclo[3.2.0]hept-2-en-6-one: (1S,5R)-2-oxa (1R,5S)-3-oxa	 37% 34%	 *>98%* >98% (See table 2.9)	(Combined lactones) 130 77.9 9.7

Table 2.8: Bioconversion properties for 5mM of substrates of increasing ring size.

Bicyclo[3,2,0]hept-6-en-2-one as a racemic mixture, produces four possible isomers. The two different stereoisomers predominantly produced are shown in Figure 2.3. Whilst (1R,5S)-3-oxa-bicyclo[3,3,0]oct-6-en-2-one was produced with >98% ee, that of (1S,5R)-2-oxa-bicyclo[3,3,0]oct-6-en-3-one appears to be dependent upon the substrate concentration as is shown in Table 2.9. It has not escaped notice that this may explain the difference in *ee* seen by Doig *et al.* (2003) and Simpson *et al.* (2001) of 94% and >98% respectively for this lactone.

Ketone concentration (mM)	*ee* of (1S,5R)-2-oxa-bicyclo[3,3,0]oct-6-en-3-one (%)
5	>98%
10	98%
20	97%
30	93%
40	84%
50	70%
100	-6%

Table 2.9: The effect of substrate inhibition on the enantiomeric excess of (1S,5R)-2-oxa-bicyclo[3,3,0]oct-6-en-3-one. Note that at 100mM the true ee is actually a 6% excess of the other enantiomer (1R,5S)-2-oxa-bicyclo[3,3,0]oct-6-en-3-one.

Effect of chain length - The initial specific activity of the isolated enzyme, free whole cells and immobilised whole cells is compared at a 5mM substrate concentration in Figure 2.29. The free cell reaction rate and immobilised whole cell reaction rates appear to show significant decline for increased chain length. From Figure 2.30, where the free cell catalyst effectiveness in comparison to the isolated enzyme reaction rate is shown, the effect of increasing chain length is an almost linear reduction in whole cell catalyst effectiveness. This linear relationship is further described in Figure 2.31, from which it can be seen that for each carbon bond added to the chain length roughly a 20% reduction in reaction rate is seen. The cause of this must be a linear reduction in favourable physical properties of the substrate, which solely affects the mass transfer rate and has no effect on the catalyst active site.

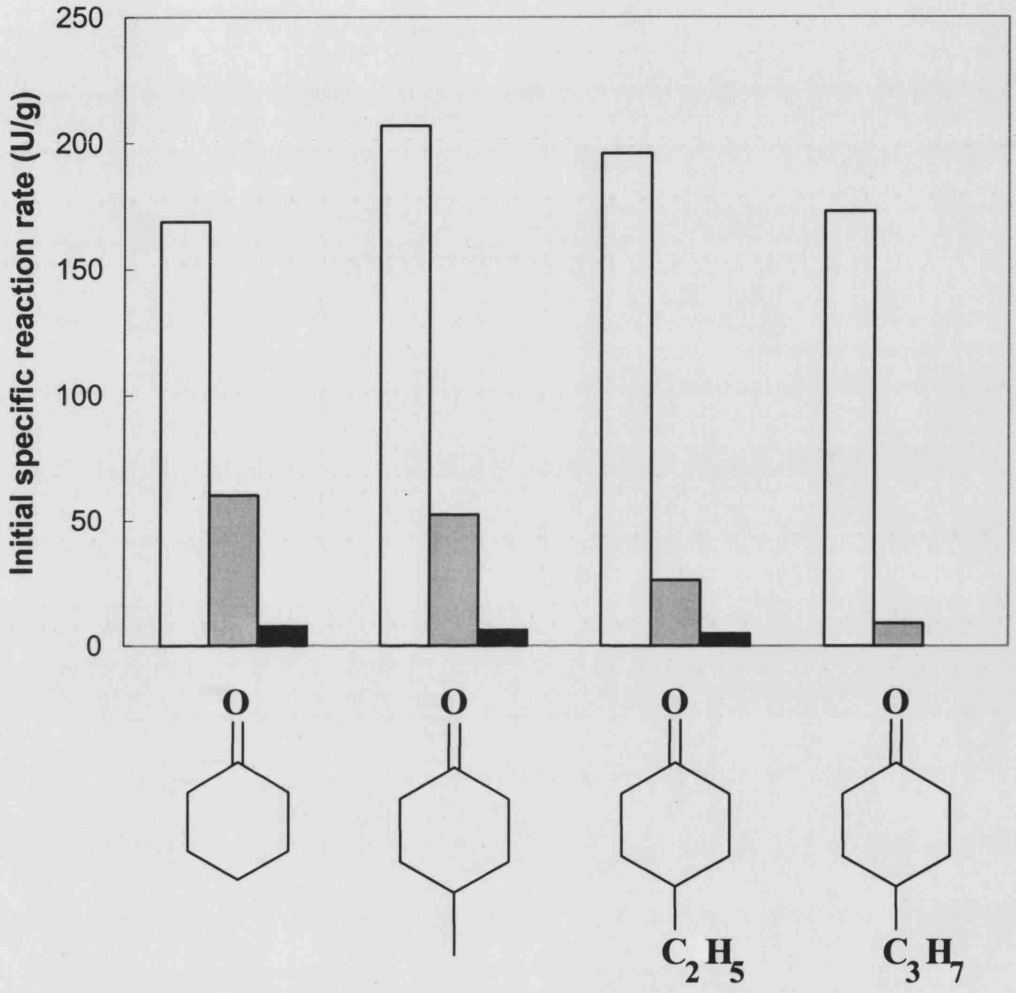


Figure 2.29: Effects of chain length on a comparison of the initial specific activity of isolated enzyme, whole cells and immobilised whole cells of E. coli TOP10 [pQR239] for the oxidation of (left to right) cyclohexanone, 4-methyl cyclohexanone, 4-ethyl cyclohexanone and of 4-propyl cyclohexanone.

Where:

□ Isolated enzyme, ▒ Free cells, ■ Immobilised whole cells

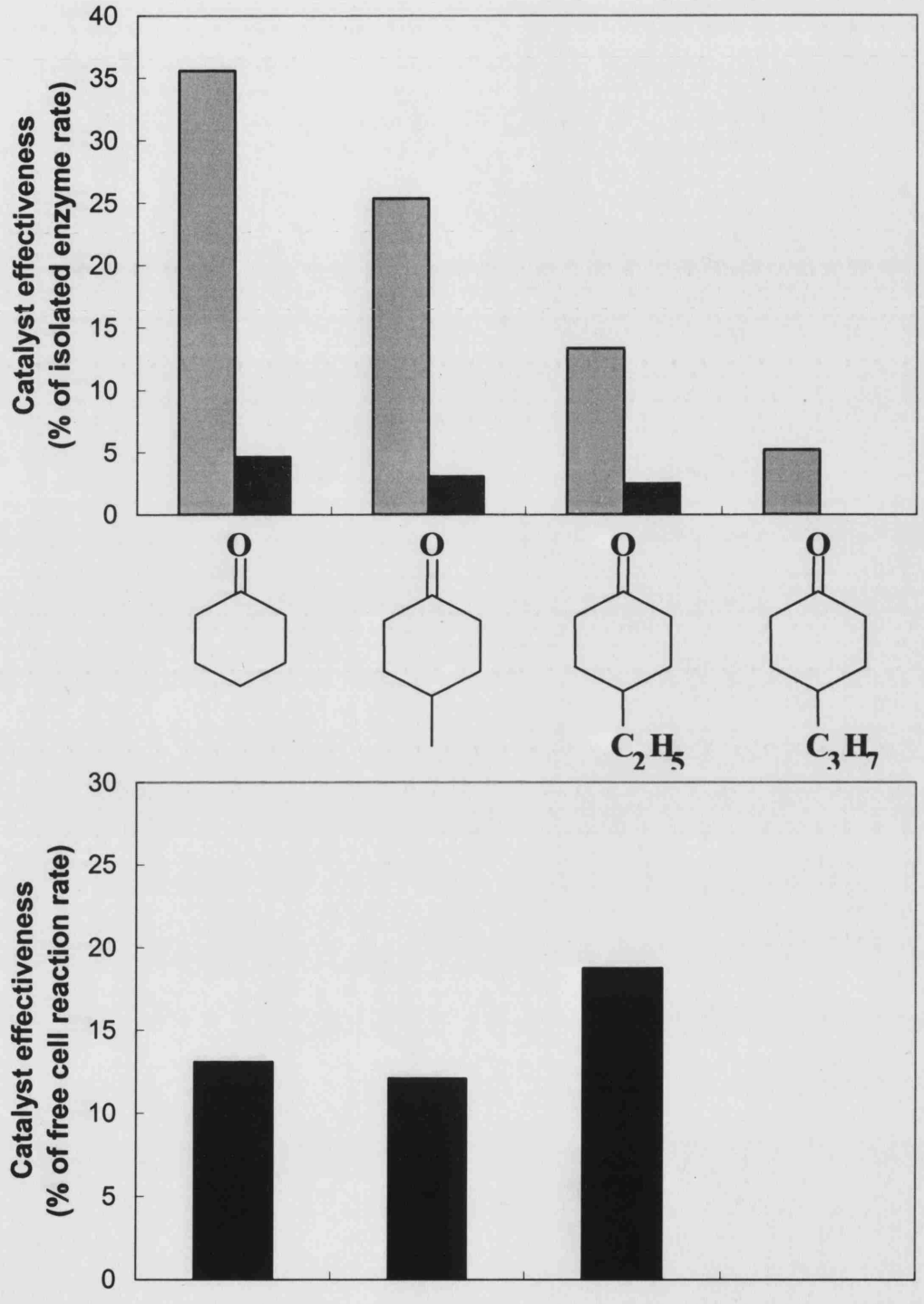


Figure 2.30: Effects of chain length on a comparison of the initial specific activity of whole cells and immobilised whole cells of E. coli TOP10 [pQR239] for the oxidation of (left to right) cyclohexanone, 4-methyl cyclohexanone, 4-ethyl cyclohexanone and of 4-propyl cyclohexanone
Where: ▒ Free cells, ■ Immobilised whole cells
Top: Free and immobilised whole cell reaction rates as a percentage of isolated enzyme reaction rate. Bottom: Immobilised whole cell reaction rate as a percentage of free cell reaction rate.

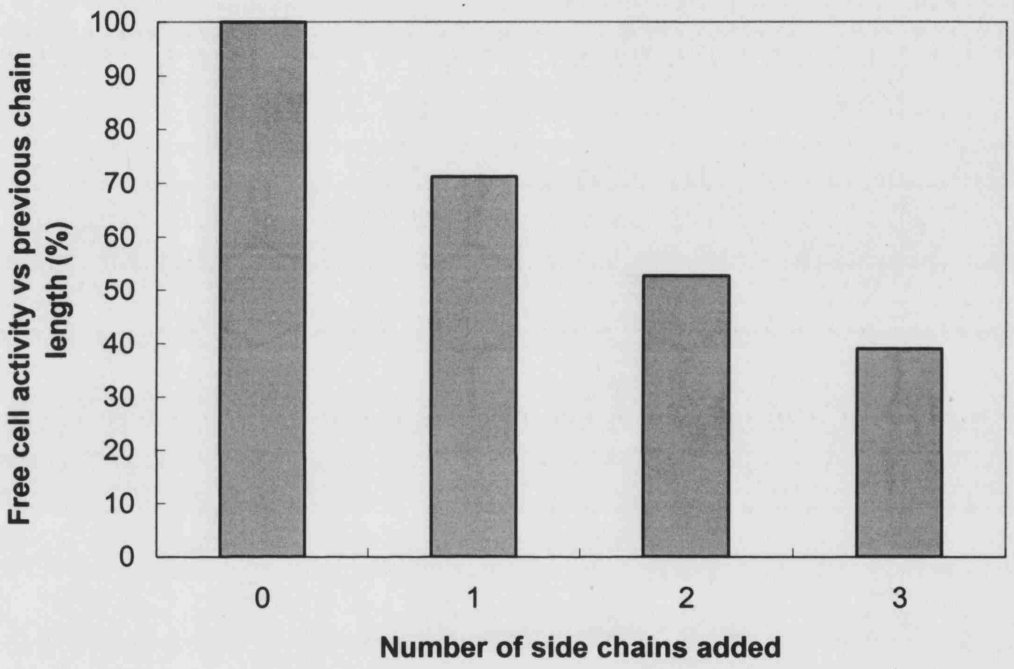


Figure 2.31: Effect of increasing the side chain length on the relative free cell activity as a percentage of the previous side chain length, e.g. 0 is the activity of cyclohexanone, 1 is the percentage reduction in activity of 4-methyl cyclohexanone compared with cyclohexanone.

By contrast to the linear free cell catalyst effectiveness, the immobilised whole cell catalyst effectiveness (comparison against free cell reaction rate) is similar for cyclohexanone and 4-methyl cyclohexanone, and is higher for 4-ethyl cyclohexanone. Whilst the former may suggest that a degree of substrate mass transfer limitation remains in the immobilised whole cells, the latter suggests a shift towards oxygen limitation.

Free cells substrate yields are similar for all substrates bar 4-propyl cyclohexanone, which again gave the slow reaction rate (Table 2.10). However, the similar yields on substrates with differing reaction rates suggests that the link between yield and reaction rate is not so close as that which was seen with increasing ring sizes. Again the product purity (*ee*) is higher than the limits of detection for all substrates studied.

Substrate	**Free cell yield (%)**	**Free cell *ee* (%)**	**Isolated enzyme, free cell and Immobilised whole cell specific reaction rate (U/g)**
cyclohexanone	80%	>98%	169 60.2 7.9
4-methyl cyclohexanone	75%	>98%	207 52.5 6.4
4-ethyl cyclohexanone	83%	>95%	196 26.2 4.9
4-propyl cycloheptanone	67%	>95%	173 9.0 0.0

Table 2.10: Bioconversion properties for 5mM substrates of increasing chain length.

Generally the effect of immobilisation appears to reduce the reaction rate to a greater extent than adding the cell membrane to the isolated enzyme for the large beads used throughout this study. For example with cyclohexanone a 36% reduction in reaction rate occurs when the whole cells are used instead of the isolated enzyme. Compared to this, immobilisation reduces the reaction rate to 13% of that of free cells. Also the inhibitory nature of the substrates actually appears to have been increased with immobilised cells rather than the expected reduction in inhibition seen.

2.7 Conclusions

2.7.1 The effect of enzyme protection on inhibition

The hypothesis proposed was that the higher the level of protection offered to an enzyme, e.g. by performing reactions in whole cells with protective cell membranes and intact metabolic pathways rather than isolated enzymes, or by subsequent immobilisation of whole cells, the less inhibitory a substrate and its products would appear. To enable the industrial use of immobilised whole cells systems, two concerns had first to be overcome:

1. That such systems yield reactions rates that are so low that they are not economically feasible. The work performed here demonstrates that immobilised whole cell oxygenase reactions are possible with only a two fold reduction in reaction rate with bicyclo[3.2.0]hept-2-en-6-one and small beads (1mm) with the potential to produce smaller beads with even more comparable reaction rates.
2. That due to the harsh cells separation conditions required for immobilised cell production the scale-up of immobilised bead formation may not be feasible. Flow cytometry and shaken flask reactions have been used to effectively demonstrate how mild the cell separation conditions have become in both cross-flow filtration and modern hydro-hermetic centrifuges. Thus with the potential scale-up equipment as suggested in Appendix VII, Figures AVII.1 and AVII.2, the scale up of immobilisation appears feasible.

Whilst the potential effectiveness of immobilised cell reactions has been shown, the inhibition profiles seen with different CHMO catalysed reactions - isolated enzyme, free whole cell and immobilised whole cell – suggest that increasingly complex systems actually increase the inhibition observed rather than provide protection to the enzyme. It is postulated that this is due to both the reduced availability of oxygen in increasingly complex systems and due to the reduced reaction rates, the inhibition seen is also a function of the time for which the enzyme is exposed to a substrate/product concentration (figure 2.32).

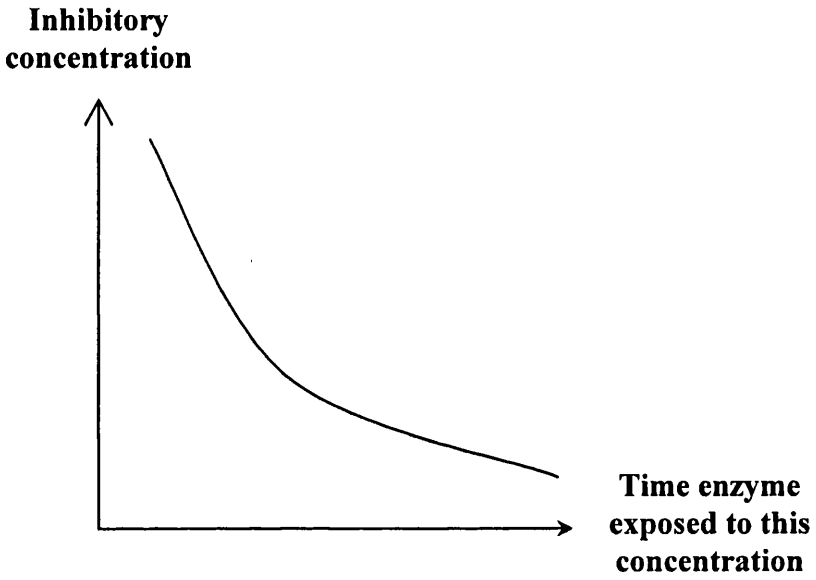


Figure 2.32 – The proposed effect of length of exposure time on the inhibitory concentration of any substrate or product.

This result could suggest that the there is little advantage to operating in an immobilised whole cell system, however the ease with which beads can be recycled and the cleanliness of the subsequent process stream may be of sufficient importance alone to merit immobilisation. It is also possible that the enzyme longevity will be increased and thus this warrants further attention.

It has not escaped notice that the high development costs of pharmaceuticals and benefit of getting new drugs to market quickly decreases the effort that is likely to be spent on process improvement. Therefore the use of immobilised whole cell

biocatalysis is unlikely to become widespread until generic competition increases and operating cost savings become significant.

2.7.2 Analysis of inhibitory concentration

Given the importance of inhibition as a key bottleneck in the increased application of biocatalysis within the pharmaceuticals development process, rapid analysis of the extent of inhibition of any given substrate/product would be beneficial. Two potentially functional indicators of inhibitory concentration have been discovered within this study:

1. From the results of the different substrates studied, it appears that by grouping similarly structured substrates together, the inhibitory substrate concentration is broadly proportional to the change in initial rate of reaction. For example in comparison with cyclohexanone, substrates with increasingly different ring size or increasing chain length have lower reaction rates and also become inhibitory at lower substrate concentrations (see figure 2.33).

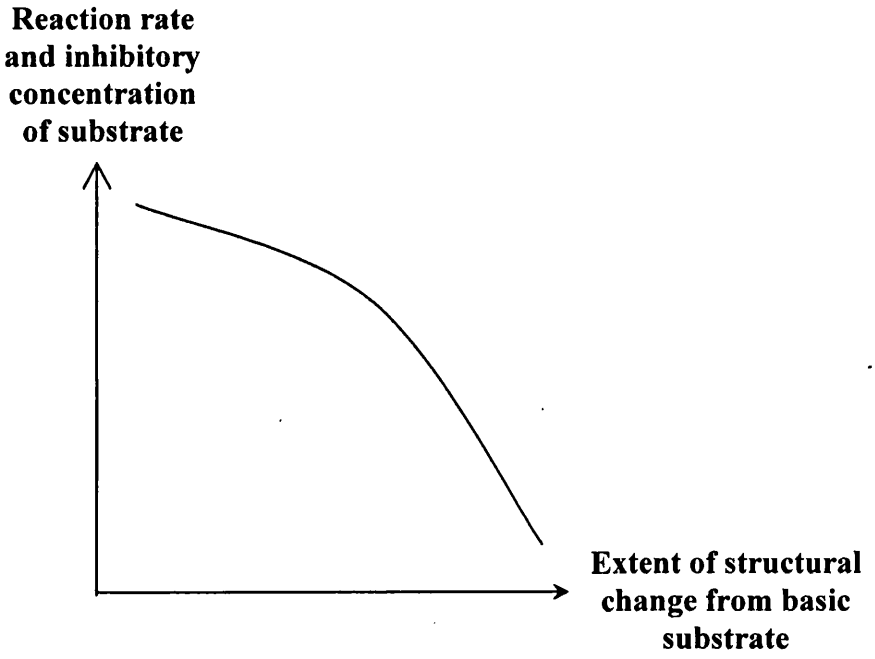


Figure 2.33 – Observed correlation between the reduction in reaction rate and the lower inhibitory concentration of a substrate in comparison with the enzyme's standard substrate.

2. Flow cytometry has shown that the toxicity of a substrate to the cell membrane appears closely related to the level of enzyme inhibition seen. This could be utilised as a useful indicator of the inhibitory level of a new substrate in comparison with a known substrate (see figure 2.34).

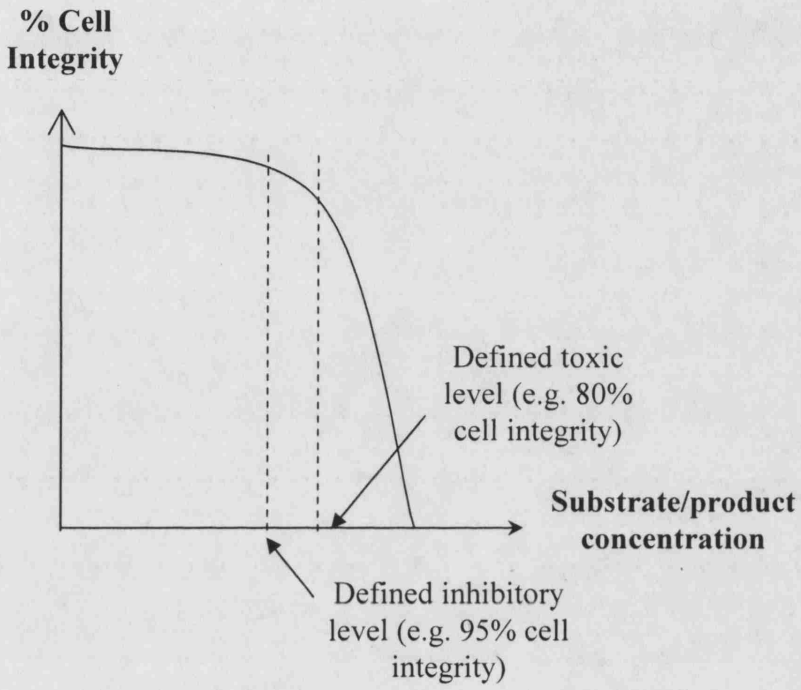


Figure 2.34 – The potential use of flow cytometry as an indicator of the notional substrate/product concentration at which enzyme inhibition occurs.

With further development both of these methods could potentially be implemented in the determination of the extent of substrate inhibition.

3 Metabolite production of drug lead candidates

3.1 Aims of the chapter

- To enable the CYP450 enzyme responsible for the metabolism of drug lead candidates to be determined.
- To assess the scale-up potential of gram quantity metabolite production.

3.2 Introduction to the scale-up of drug metabolism

The importance of ADMET studies in the development of drugs has been discussed in section 1.3. Whilst such properties have crucial impacts on the potential of a drug candidate, the properties of metabolites of such drug candidates are just as important given that such metabolites can be bioactive or toxic. Indeed with regulatory demands increasing, the ADMET properties of metabolites can have as much effect on the suitability of a drug candidate as the parent molecule that is extensively tested (Figure 3.1).

One reason why such studies are not as extensively adopted as would ideally be the case that drug metabolism studies (performed in parallel with drug development or in pre-clinical trials) produce very small quantities of the key metabolites. Scaling-up the production of metabolites would be potentially beneficial for several reasons. It would:

- Aid in the assessment of drug-drug interactions, many of which are caused by metabolites, rather than the parent molecule. Whilst much interest has been shown in this area (Bachman and Lewis, 2005; Hutzler *et al.*, 2005), it is not presently something that needs to be tested in clinical trials even though it is responsible for 100,000 deaths per year (Kremers 2002).
- Aid in the ADMET assessment of drug metabolites, some of which may be more suitable drug candidates than the parent molecules, i.e. it will allow the production of second generation drugs with preferable ADMET properties.
- Provide analytical standards, allowing more quantitative studies to be performed on the parent molecule.
- Provide new drug scaffolds, not possible under chemical synthesis.

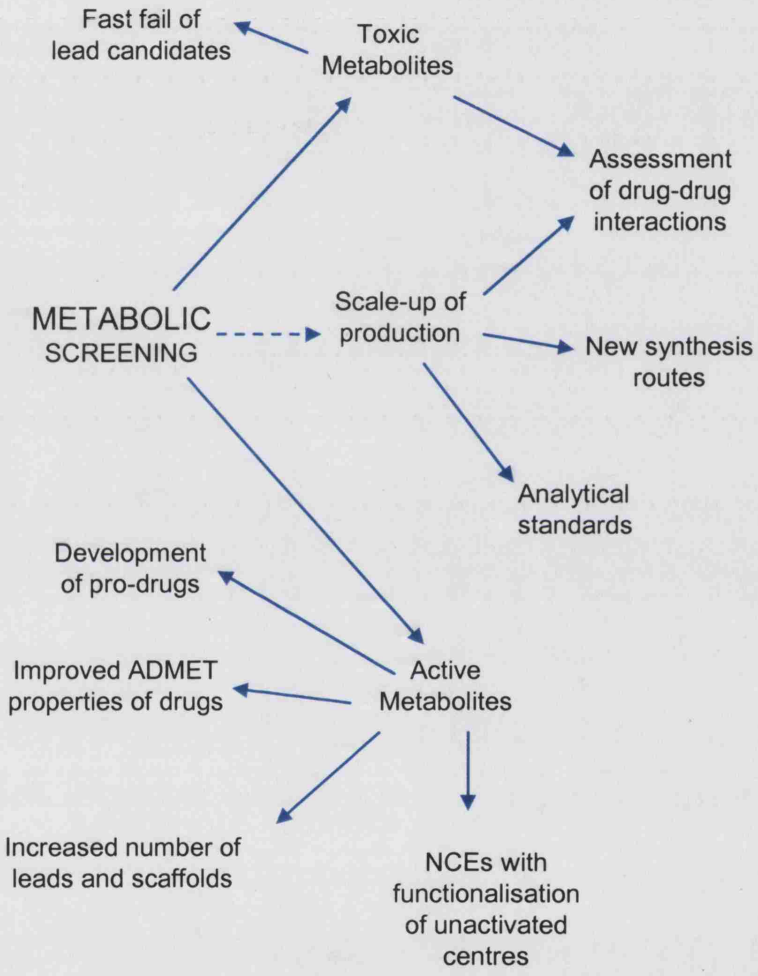


Figure 3.1: Outcomes from the scale-up of metabolic screening.

A potential route for the scale up of metabolite production is given in Figure 3.2. Initially experiments to find the metabolising CYP enzymes and the metabolites produced (metabolic profiling) would be required. This would then enable the selection of a cell line expressing the appropriate CYP and large-scale biotransformation allowing production of up to kg scale metabolite production, prior to purification of the identified metabolites. Each stage, where possible, is experimentally tested and discussed below (sections 3.5 to 3.7).

Three types of inhibition have been described for CYP3A4 and various models from single to multiple reactive sites have been proposed to account for the relatively poor predictive capability of mathematical models. *In vitro* approaches to studying metabolism generally focus on finding the predominant CYP enzyme and studying the resultant inhibition. The inhibitory effect of the metabolites of a drug-candidate is often only studied *in vivo* due to the potential difficulty with which they are generated. Whilst different methods have been published for assessing the metabolizing enzyme, they have yet to be compared. In this study, the comparison of the different techniques will be made.

3.3 Materials and methods

3.3.1 Reagents and suppliers

The microsomes and the NADPH Regenerating Solution (NRS) were obtained from two sources: *In vitro* technologies (Baltimore, US) and BD Biosciences (San Jose, US). Bactosomes™ were obtained from Cypex (Dundee, Scotland). All other reagents used were of analytical grade and were obtained from Sigma (Poole, UK).

3.3.2 Storage of CYP host cells

Microsomes and Bactosomes™ were shipped on dry ice and subsequently stored in liquid nitrogen (-80°C).

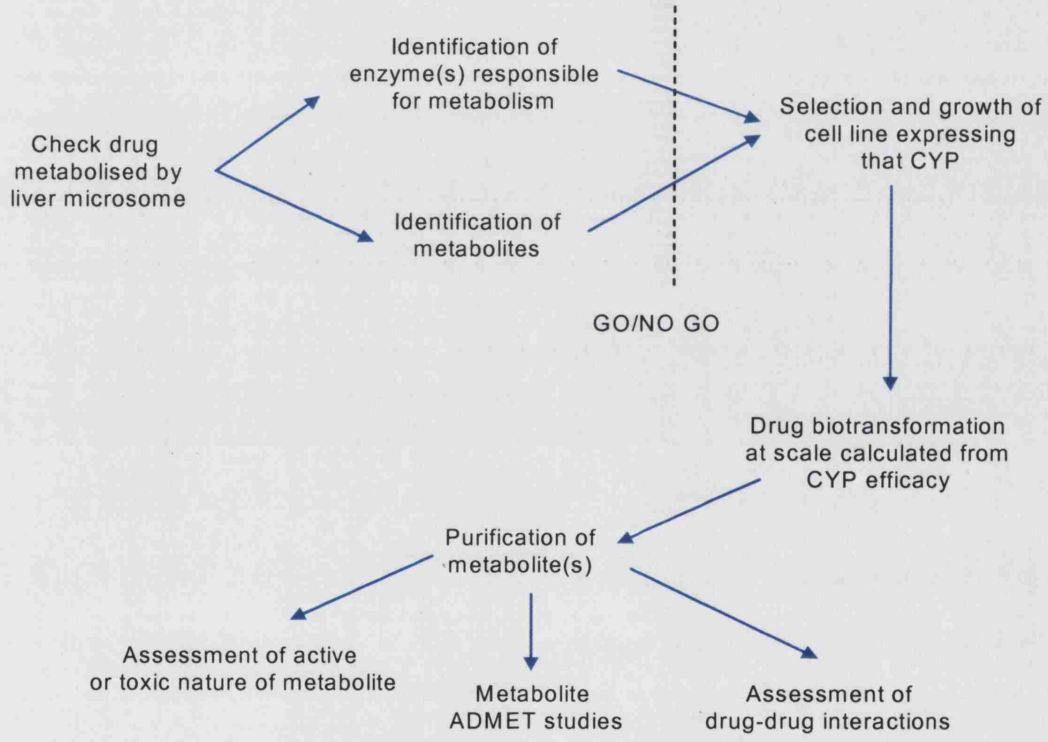


Figure 3.2: A potential route to scale-up and metabolite production.

3.3.3 Metabolism assays

3.3.3.1 Pre-experimental preparation of reagents

pH 7.4 potassium phosphate buffer (PPB) (1.63g K_3PO_4, 1mL H_3PO_4) was prepared fresh weekly.

NRS was prepared no more than eight hours prior to each experiment (as per the microsomes and bactosomes™ supplier standard operating procedures) from 62.4mg glucose-6-phosphate, 10.4μL (twelve units) glucose-6-phosphate dehydrogenase, 13.6mg $NADP^+$ and 160mg $NaHCO_3$ (equating to 8mL of 2% w/v solution).

Test drug solutions were prepared as required prior to each experiment: 5-10 mg of test drug was dissolved in 0.5mL acetonitrile (ACN) and diluted in water to 0.3mM.

Individual vials of microsomes and bactosomes™ were thawed on ice as required.

3.3.3.2 Experimental Procedure

A ninety six-well incubation plate was put on ice and, for a standard reaction, 50 μL microsomes, 10 μL drug solution (3μM final well concentration) and 690μL PPB (to give a final well volume of 1mL after NRS addition) were added. Individual wells were pipette-mixed (500μL volume) three times and the lidded incubation plate was warmed in water bath (37°C, 150 rpm) for five minutes.

A 75μL (t=0) sample was taken from each well and added to the 100μL ACN and the 25 μL PPB (to account for the lack of NRS volume in the initial sample) in the sampling plate. Then 250 μL NRS solution (pre-warmed with the incubation plate) was simultaneously added to the eight reaction wells to start the reaction. For the inhibitor studies, ketoconazole was added to the incubation plate with the NRS. The final well concentration of inhibitor was 1mM, based on work by Crespi *et al.* (1997).

100µL samples were taken (according to the schedule in Table 3.1) from the incubation plate and the reaction quenched by adding to 100µL ACN and pipette-mixed three times.

Sample	1	2	3	4	5	6	7	8	9
Time (minutes) $T_{1/2}$	0	1	3	6	9	12	15	30	45
Time (minutes) T_{Met}	0	2	5	9	14	20	30	60	120

Table 3.1: Sampling time points used for the metabolism assays.

Where: $T_{1/2}$ are the time points used for half-life calculation assays

T_{Met} are the time points used for metabolite ID and active enzyme ID

After the ninth sample, the sample plate was centrifuged in a Qiagen (Crawley, UK) 4K15C centrifuge for ten minutes at 3000rpm and 5°C to ensure samples were clean and solids-free prior to analysis by LC-MS/MS.

3.3.4 LC-MS/MS Analysis

High-pressure liquid chromatography-tandem mass spectrometry, through its high sensitivity and selectivity, permits the use of very low incubation concentrations of microsomal protein (0.01-0.2 mg/ml). Walsky and Obach (2004) found that its analytical assay accuracy and precision values were excellent for use in metabolism studies.

Samples were analyzed by staff at EvotecOAI on a HP1100 Series Liquid Chromatograph (Hewlett-Packard, Palo Alto, CA, USA) interfaced to a Micromass (Manchester, UK) quadrupole time of flight mass spectrometer with W-optics. 10µL from each sample was injected onto a Phenomenex Luna C18(2) column (50mm x 2.0 mm. 5µm particle size). The flow rate was 1 ml/min. Mobile phase A was acetonitrile and mobile phase B was 0.1% formic acid in water. Mobile phase A was linearly ramped from 0 to 50% in 3 minutes, held at 50% for an additional 0.5 minutes, and then immediately stepped back down to 0% for re-equilibration (total run time 5.5

minutes). After 1.5 minutes, the LC eluent was diverted from waste to the mass spectrometer fitted with electrospray ionization (ESI) source and operated in the positive ion mode. The LC flow was split so that approximately 150 µl/min entered the mass spectrometer. The needle voltage was set to 4.5 kV and the sheath and auxiliary gas flows to 80 and 20 (arbitrary units) respectively. The capillary heater temperature was maintained at 325°C and the source manifold at 70°C. The argon gas pressure in the collision cell was approximately 2 mTorr. For quantification, the mass spectrometer was operated in the selected reaction monitoring (SRM) mode to monitor for metabolites with a dwell time set to 0.1 seconds for each reaction.

Metabolites produced were found and analysed using MetaboLynx™ software (Micromass, Manchester, UK), which automates the long and laborious process of data interpretation (Castro-Perez and Preece, 2001).

Due to the metabolites not being commercially availabile, plots of metabolism are either shown as a percentage of the initial amount or where the metabolites produced are shown the arbitrary units of mass spectrometer peak area are used. Peak area is not proportional to the concentration of each shown and thus whilst the profiles of each metabolite produced is representative, the different amounts of each metabolite cannot be compared.

3.4 Initial results

3.4.1 Selection of drug candidate

The solubility of drug compounds is a known problem in the analysis of drug metabolism, with research into the effects of different solvents at various concentrations having been carried out. From the results of Busby *et al.* (1999) and Chauret *et al.* (1998) the best solvent, i.e. the one that had the least effect on the microsomal activity, is acetonitrile used at low concentrations (<0.3%). Therefore this was the solvent and maximum concentration used for dissolving the drugs studied.

Verapamil, an antihypersensitive, is a clinically important inhibitor of CYP3A4 (Zhou *et al.*, 2005) and is known to be rapidly metabolised by both CYP3A4 and CYP1A2 (http://medicine.iupui.edu/flockhart). Due to these known metabolism properties, known metabolites (Table 3.2 and Figure 3.3), available selective inhibitors and reasonable solubility (allowing good detection by LC-MS/MS) it was selected as a suitable candidate for analysis of metabolite scale-up potential.

Compound	Molecular Formula	Molecular Weight
Verapamil	$C_{27}H_{38}N_2O_4$	454.28
Norverapamil	$C_{26}H_{36}N_2O_4$	440.26
D-617	$C_{17}H_{26}N_2O_2$	290.20
D-620	$C_{16}H_{24}N_2O_2$	276.18
D-703	$C_{26}H_{36}N_2O_4$	440.27

Table 3.2: Molecular formulas and weights of verapamil and its metabolites.

Diltiazem was chosen as a secondary drug to study in parallel, though this was used to a far lesser extent than verapamil as it has only one known metabolite (Desacetyldiltiazem).

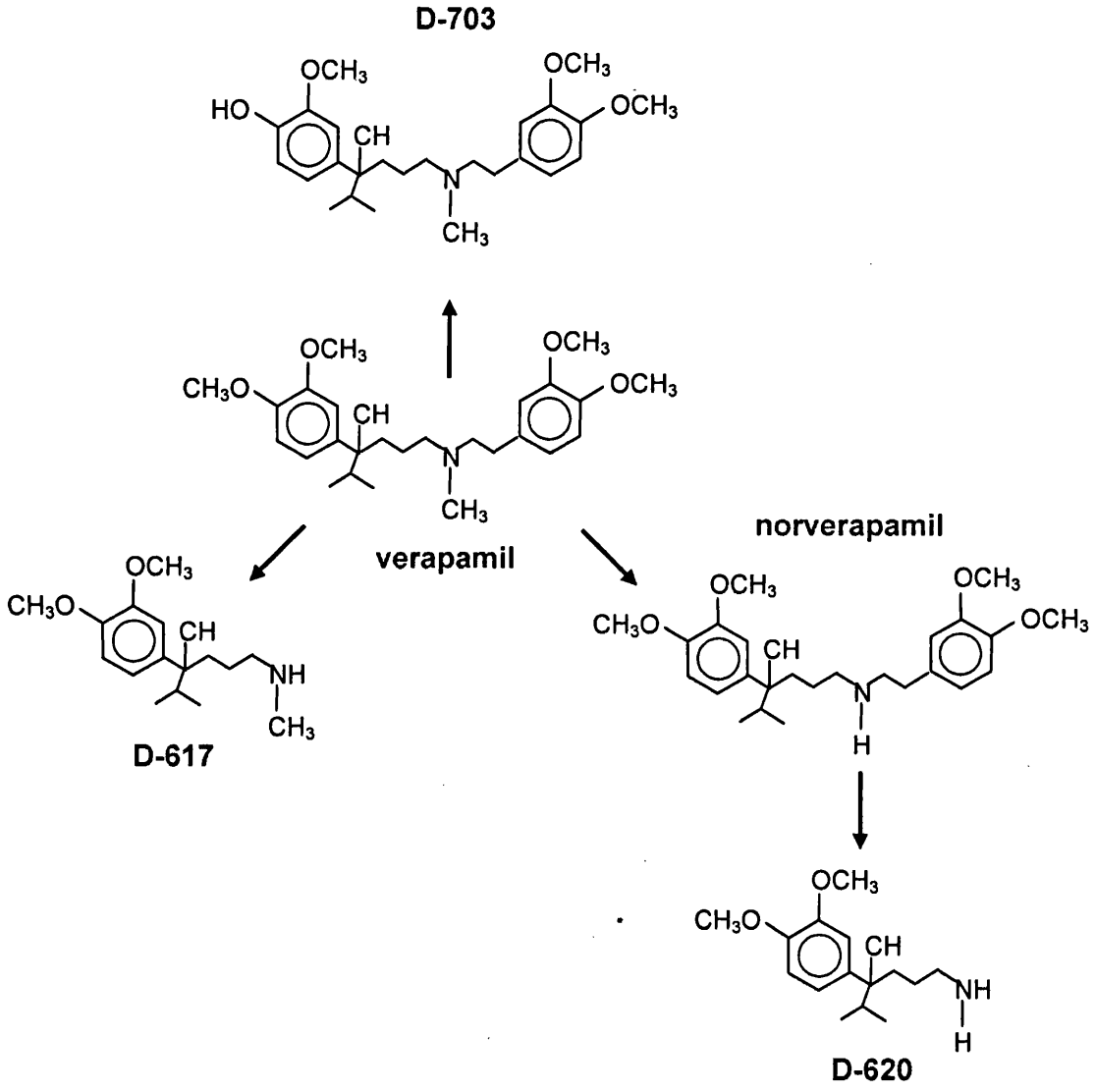


Figure 3.3: Verapamil and its metabolites, modified from Eichelbaum *et al* (1979).

3.4.2 Selection of liver microsomes and NRS

There are two sources of both liver microsomes and NRS:

- *In vitro* Technologies
- BD Biosciences

The difference between sources of materials is studied in Chapter 5, where the effect of changing suppliers on a validated assay is assessed. From these results BD Biosciences microsomes and the IVT method of freshly preparing NRS was selected.

3.5 Stage 1: Metabolism of the drug candidate and metabolite identification

Drug metabolism is traditionally studied in the liver microsomes of humans, rats, dogs or monkeys (section 1.3.4.2) and yields a variety of metabolites in concentrations partially dependent upon the concentration of each CYP enzyme. Metabolite identification without any knowledge of the metabolites can be a long and laborious process, and generally requires expertise in being able to guess likely metabolites and their molecular weights from the possible reactions, e.g. epoxidation, hydroxylation, and demethylation (Jiang and Morgan, 2004). MetaboLynx™ is a relatively new software package, which automates the process of identifying potential metabolites and quantifying their production.

Figure 3.4 gives a typical profile of the metabolism of verapamil and the generation of its metabolites. The same molecular weights of norverapamil and D-703 make these metabolites impossible to separate by mass spectrometry and the reported figures combine these metabolites. It was reported by Eichelbaum *et al.* (1979) that D-620 is a metabolite of norverapamil. Figure 3.4 shows that no D-620 is produced until after four minutes which supports this fact.

As different drugs are metabolised at different rates, it is important that the system is comparable for different substrate (drug) and microsomal concentrations to enable the metabolism to be studied at different conditions and therefore compared fairly.

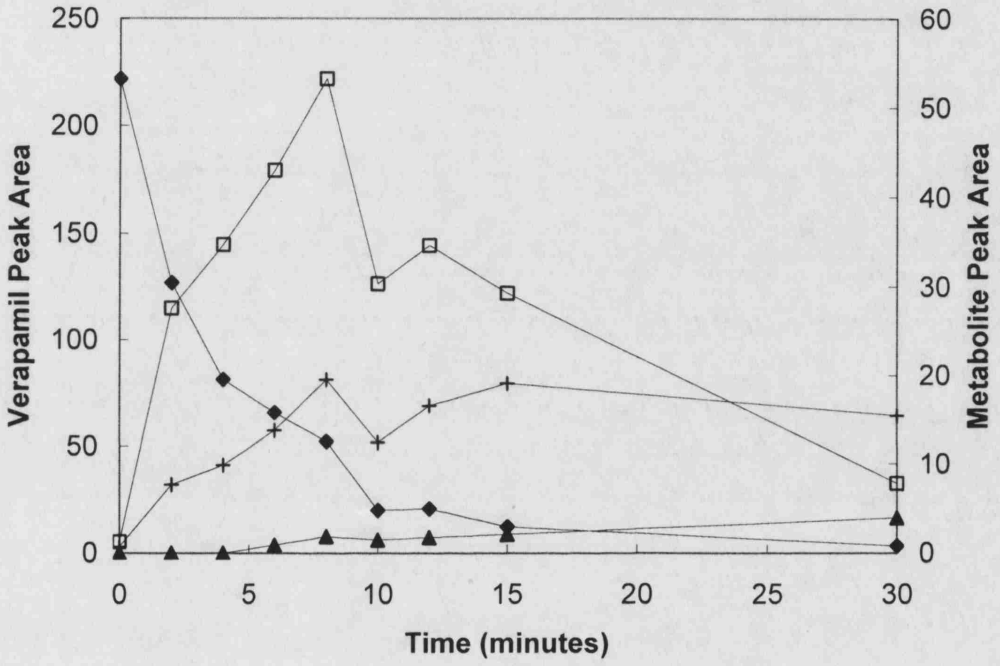


Figure 3.4 Metabolites of verapamil from BD Biosciences rat liver microsomes and fresh in vitro technologies NADPH regenerating system.

◆ Verapamil, □ Norverapamil & D-703, + D-617, ▲ D-620

Figure 3.5 shows the effect of metabolising different concentrations of verapamil. By comparing the percentage remaining it is possible to vary the apparent rate of metabolism, making it more easily studied. Figure 3.6 shows the production of the metabolites of verapamil at the different drug concentrations. These graphs illustrate how the initial concentration of verapamil could be controlled to enable the production of a given metabolite, for example if norverapamil or D-703 were the metabolites of which kilogram quantities were required, then a high concentration (30μM) of verapamil with the reaction quenched at 30 minutes would be optimal.

Figure 3.7 shows the half-life of verapamil for the different concentrations. From this linear plot it appears that the half-life is proportional to verapamil concentration. Due to the speed at which 1.5μM of verapamil is metabolised the calculation of the half-life is less accurate than at higher concentrations; the expected half-life being around 1.5 minutes rather than the slightly higher calculated 2.3 minutes. This result is due the relatively few time points used in the calculation (two time points) and highlights the potential problem of using drug concentrations that are too rapidly metabolised.

The proportional metabolism of different concentrations of verapamil would allow different concentrations of separate drugs to be directly compared and would allow control over the apparent rate of metabolism.

A method of varying the actual rate of metabolism is by using different microsomal concentrations. Figure 3.9 shows the effect of this on this disappearance of verapamil and Figure 3.10 shows the metabolites produced with different microsomal concentration. Figure 3.8 shows the half-lives of the different microsomal concentrations. For the three concentrations studied, the rate of metabolism and thus the half-life is almost linear. However there does appear to be a slight relative increase in the reaction rate at lower microsomal concentrations.

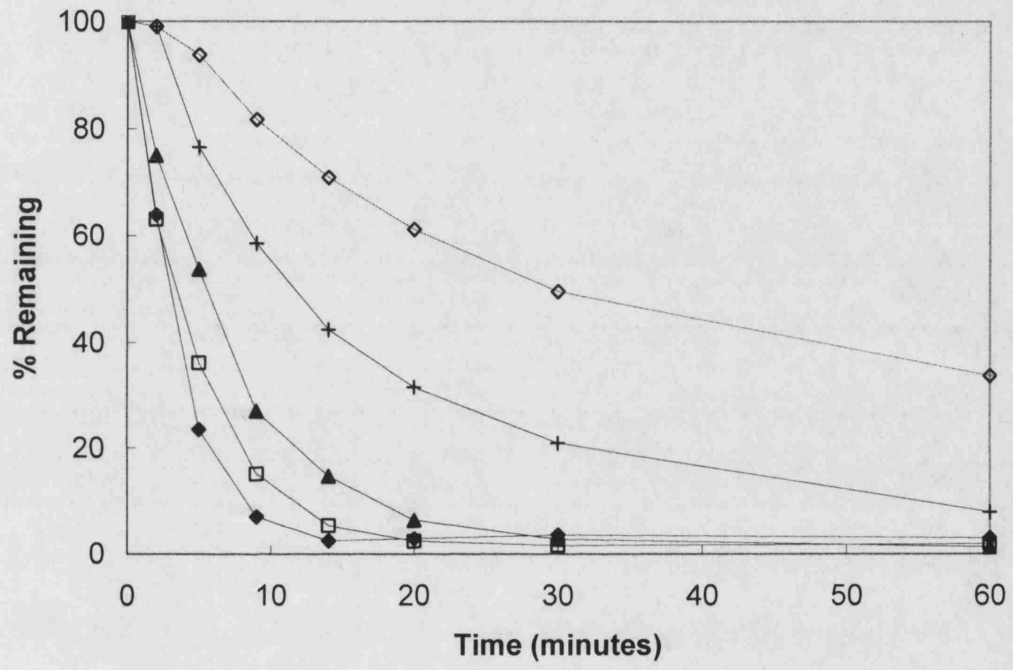


Figure 3.5: Metabolism of various verapamil concentrations by 50µL BD Biosciences rat liver microsomes and fresh IVT NADPH regenerating system, where:

◆ 1.5µM verapamil, □ 3µM verapamil, ▲ 6µM verapamil, + 15µM verapamil, ◇ 30µM verapamil

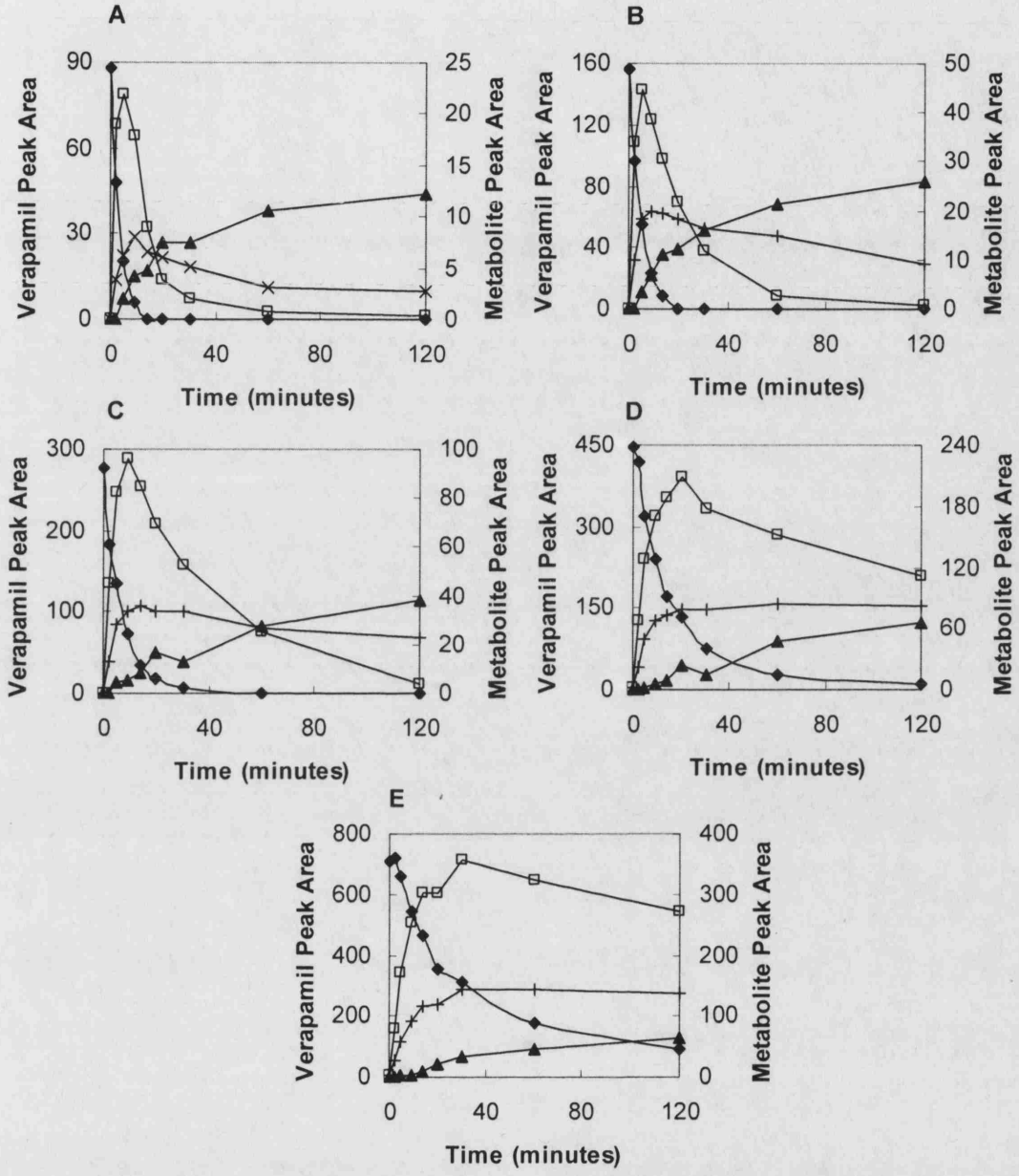


Figure 3.6: Metabolism of 1.5 (A), 3 (B), 6 (C), 15 (D) and 30 μM (E) of verapamil by BD Biosciences rat liver microsomes and fresh *In vitro* technologies NADPH regenerating system. Where:

◆ Verapamil, □ Norverapamil & D-703, + D-617, ▲ D-620

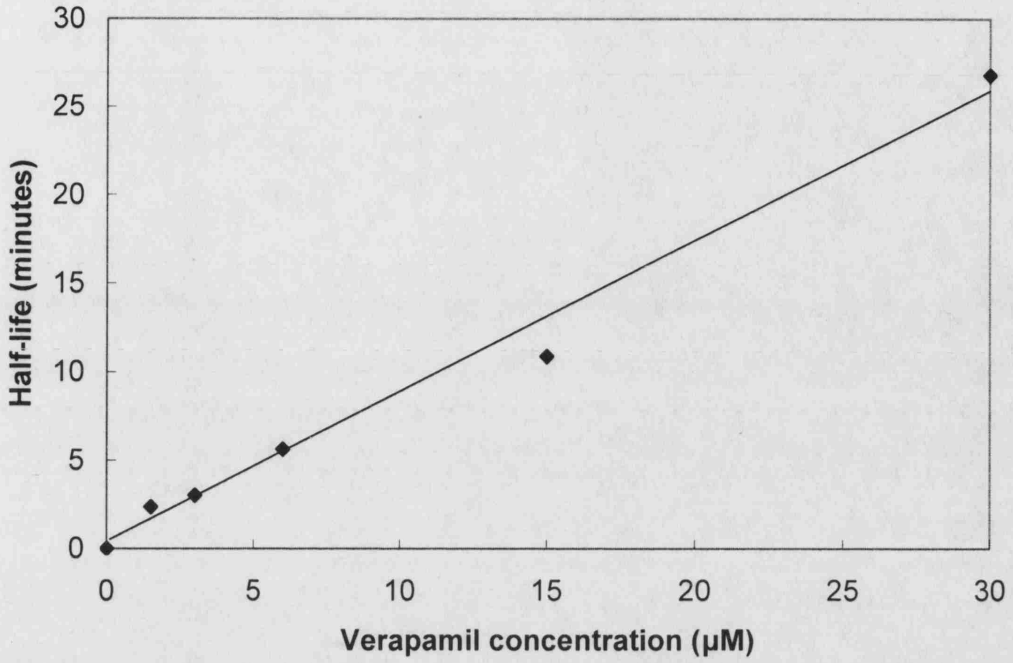


Figure 3.7: Effect of verapamil concentration on its half-life

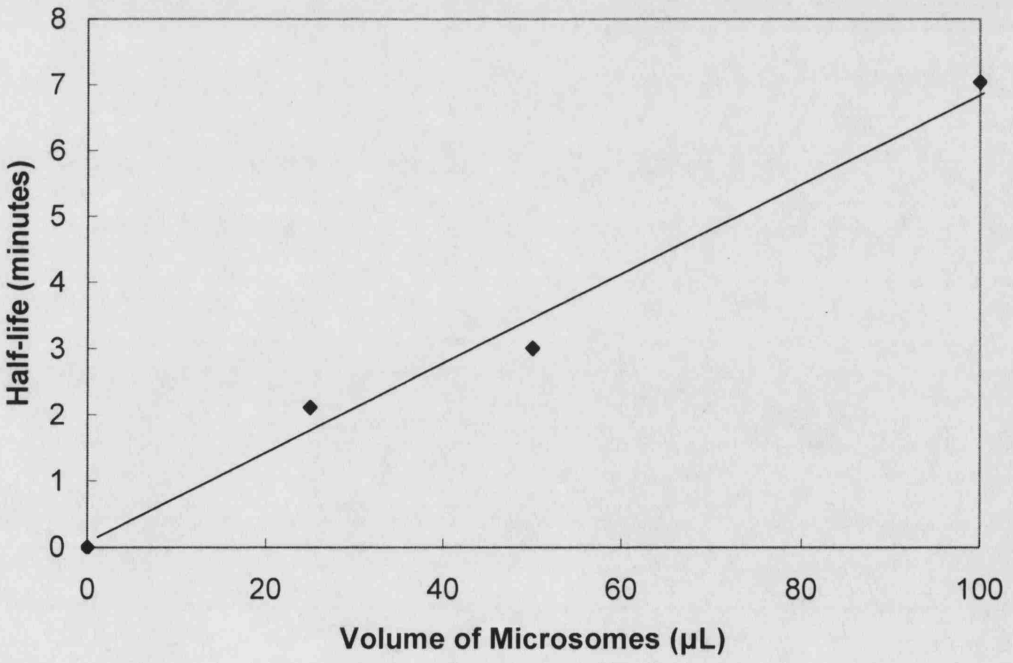


Figure 3.8: Effect of microsomal concentration on verapamil half-life

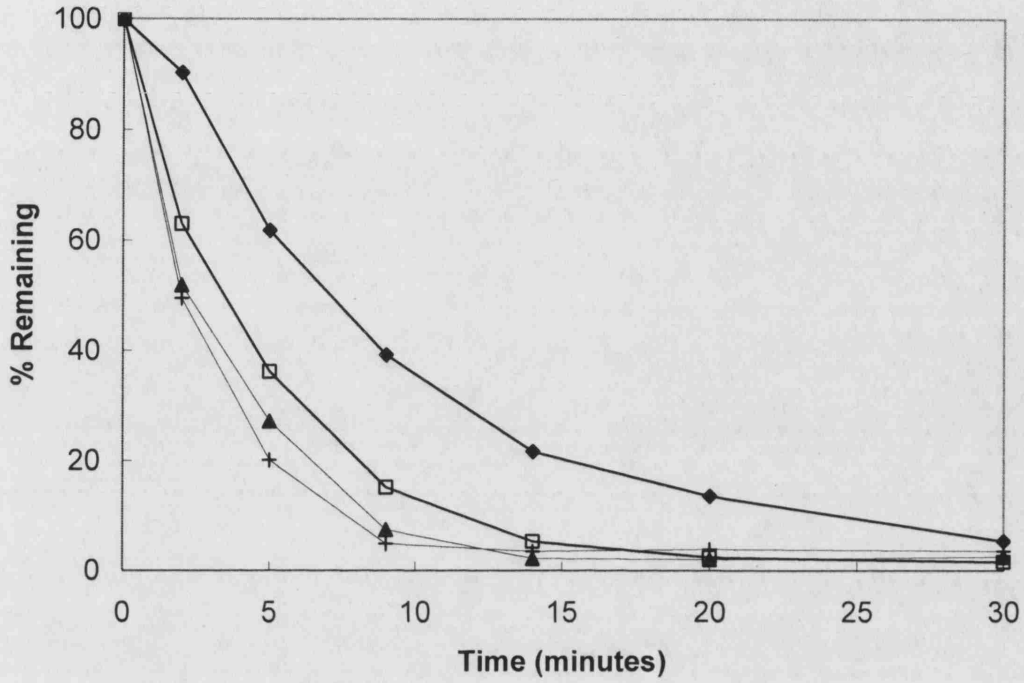


Figure 3.9: Metabolism of verapamil (3μM) by various concentrations of BD Biosciences rat liver microsomes and fresh IVT NADPH regenerating system.

◆ 25μL rat liver microsomes, 250μL NADPH regenerating system

□ 50μL rat liver microsomes, 250μL NADPH regenerating system

▲ 100μL rat liver microsomes, 250μL NADPH regenerating system

+ 100μL rat liver microsomes, 500μL NADPH regenerating system

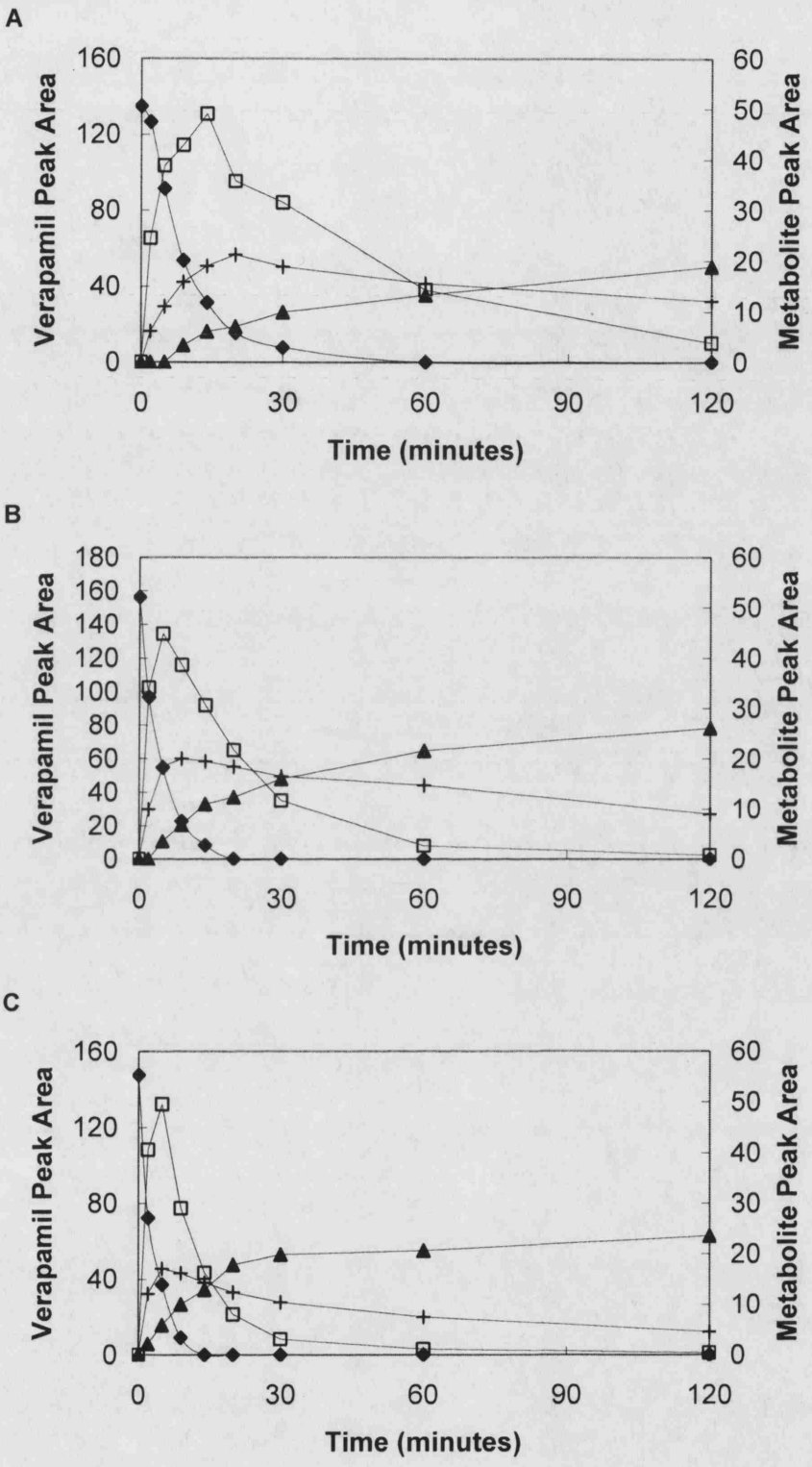


Figure 3.10: Metabolism of verapamil (3μM) by 25μL (A), 50μL (B) and 100μL (C) of BD Biosciences rat liver microsomes and fresh *In vitro* technologies NADPH regenerating system (250μL). Where:

◆ Verapamil, □ Norverapamil & D-703, + D-617, ▲ D-620

The effect of using double the concentration of NRS at the highest microsomal concentration is also shown in Figure 3.9. From this it appears that at the highest concentration the rate may be very slightly limited by the availability of NADPH. The linearity of the effect of microsomal concentration on the rate of metabolism is demonstrated further in Figure 3.10 by the point where the metabolite profiles cross. For 25μL, 50μL and 100μL of microsomes this point occurs at around sixty, thirty and fifteen minutes respectively.

3.6 Stage 2: Identification of the metabolising CYP450 enzymes

Identifying the enzyme is a key step, as knowing which CYP450 enzymes is responsible for the metabolism enables the selection of a cell host expressing the corresponding CYP. This can then be grown for the biotransformation of the drug at scale. Further key information that this provides is the likelihood of drugs interacting with one another, with most drug-drug interactions being as a result of two drugs being metabolised by the same enzyme.

Assessment of which CYP450 isozymes are functional in the metabolism of drugs by humans is carried out *in vitro*. In their review of the present status of CYP *in vitro* screening, Yan and Caldwell (2001) described the following alternative methods of identifying the CYP metabolising enzyme of different drugs:

1. Metabolism by microsomes derived from cDNA-expressed enzyme, e.g. Bactosomes™ and Supersomes™.
2. Use of selective inhibitors with microsomes, e.g. ketoconazole for the inhibition of CYP3A4.
3. Immunoinhibition of isoform specific CYP450 antibodies in microsomes.
4. Correlation of drug candidate metabolites formation with several isoform specific CYP450 activities in a panel of liver microsomes, e.g. using rat and human microsomes and assessing if the change in drug metabolism is proportional to the change in a specific CYP450 activity.

Whilst these methods have each been used, no study comparing the different methods has been found. The feasibility of the first, second and fourth of these methods has been tested below. Method three could not be compared due to the lack of commercial availability of the CYP antibodies.

3.6.1 Correlation of metabolites formation to P450 activities in a panel of liver microsomes.

The liver microsomes of individual species have significantly varied levels of the different metabolising CYP enzymes. By using a panel of such microsomes it should be possible to compare the differences in the speed of metabolism to the different concentrations of CYP enzymes present. For example CYP3A4 is known to be the predominant metabolising enzyme of verapamil, so the percentage increase in CYP3A4 concentration in one species of microsomes compared with another should yield an equivalent increase in reaction rate.

Figure 3.11 shows the metabolism of verapamil and diltiazem in human and rat liver microsomes. Both diltiazem and verapamil appear to be more rapidly metabolised in rat liver microsomes than human liver microsomes. As both of these drugs are predominantly metabolised by CYP3A4, for this method to work it would therefore be expected that there would be a higher concentration of this enzyme in rat liver microsomes. Table 3.3 gives the approximate half-lives of verapamil and diltiazem, from which it could be drawn that approximately a two to four-fold increase in concentration of CYP3A4 would be expected in rat liver microsomes compared to the human counterpart.

Conditions	Half-life (minutes)
Verapamil, BDG Rat, Fresh IVT NRS	3
Verapamil, BDG Human, Fresh IVT NRS	6
Diltiazem, BDG Rat, Fresh IVT NRS	3
Diltiazem, BDG Human, Fresh IVT NRS	13

Table 3.3 Half-lives of verapamil and diltiazem in rat and human liver microsomes

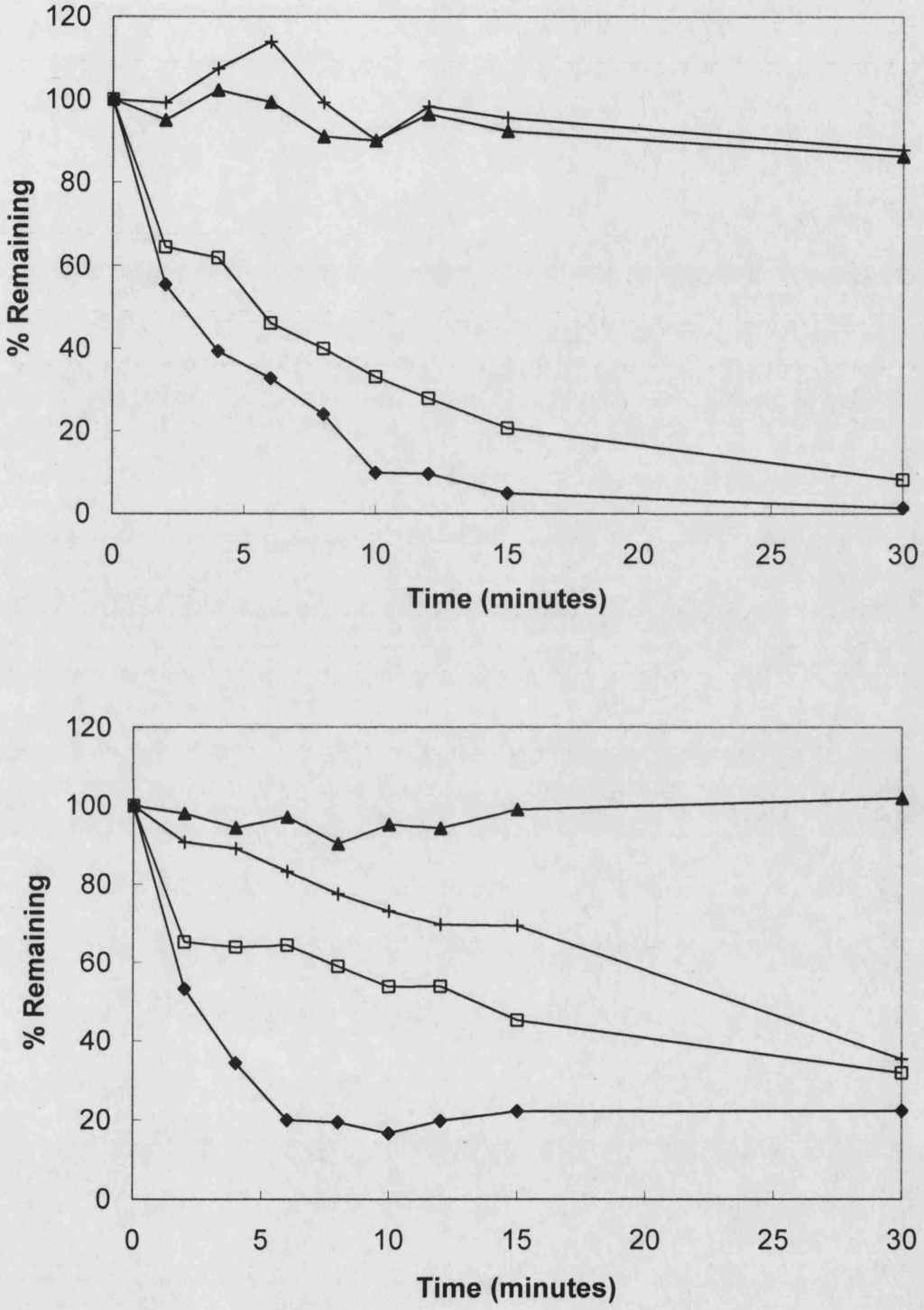


Figure 3.11: Metabolism of 3μM verapamil (Top) and 3μM diltiazem (Bottom) in BD Biosciences human and rat liver microsomes with fresh *In vitro* technologies NADPH regenerating system. Where:

◆ Rat liver microsomes, □ Human liver microsomes, + Rat liver microsomes, no NRS, ▲ Human liver microsomes, no NRS.

Table 3.4 gives the CYP concentrations in the batches of microsomes used for this study. It is immediately obvious that rat microsomes are less well characterised than human microsomes. This is due to the relative cost of the two microsomes and the fact that human microsomes are more likely to be used in later stage testing of drug metabolism, where more accurate knowledge of the CYP concentrations may be required. Whilst this lack of characterisation makes this comparison somewhat difficult, in rat liver microsomes the concentration of CYP3A (i.e. predominantly the sum of CYP3A4, CYP3A5 and CYP3A7) is lower than that of CYP3A4 in human microsomes. This shows the current lack of feasibility of this method and if used in isolation a CYP2C enzyme would appear more likely to be responsible for the metabolism. The accuracy of this method would be improved by having both better characterised microsomes and by using a more diverse range of microsomes, i.e. using dog, mouse, pig and monkey liver microsomes (all commercially available).

CYP	Human microsomes	Rat microsomes
CYP1A2	850	100
CYP2A6	690	
CYP2B6	15	
CYP2C8	140	
CYP2C9	2400	
CYP2C19	60	
CYP2D6	81	
CYP2E1	2000	800
CYP3A4	3400	
CYP4A	1300	
CYP3A		2500
CYP2C		3600

Table 3.4: The different CYP concentrations (pmol/(mg x min)) in the batches of human and rat liver microsomes used in this study, as calculated by the supplier of microsomes for each batch.

An added potential difficulty associated with this technique is the high amount of variation seen with different microsomes. Snawder and Lipscomb (2000) found that

they can vary widely depending upon the drugs being taken at the time of death and the enzyme levels they induce, e.g. CYP1A up to thirty six-fold and CYP3A up to twenty two-fold inter-batch variation on forty human microsomes samples. However, the majority of variation seen between different livers is between different ethnic populations (Oscarson, 2003; Lamba *et al.*, 2002). Such variations can be overcome by using a pooled source of microsomes (as used in this study) to ensure that all CYPs are present. Furthermore, variations between different batches of microsomes is not in itself problematic (each batch is supplied with a breakdown of the different CYP protein concentrations), however it does add to the amount of data processing required and may lead to microsomes of different species exhibiting similar CYP levels and further lessening the effectiveness of this CYP identification technique.

3.6.2 Bactosomal metabolism

Bactosomes™ are bacterial membranes containing human cytochrome P450s coexpressed with human NADPH-cytochrome P450 reductase (www.Cypex.co.uk). As they express only a single CYP enzyme, if the drug tested is metabolised by the bactosomes™ used, then the CYP responsible for the metabolism of that drug has been found.

Figure 3.12 shows the CYP3A4 bactosomal metabolism of verapamil, at different drug and bactosomal concentrations. 25µL of bactosomes (from the included characterisation) contains an equivalent concentration of CYP3A4 as that seen in 50 µM of rat microsomes, therefore all things being equivalent a similar rate of metabolism would be expected as that seen for 3µM of verapamil in Figure 3.11. However this was not the case and approximately a three-fold increase in the rate of metabolism was seen with the bactosomes™. As the bactosomes™ are non-viable when shipped, it is likely that the increase has resulted due to lack of a permeability barrier, i.e. a ruptured cell membrane. It could also potentially be a result of over-expression of the human CYP reductase in the bactosomes™. Cypex have shown (www.cypex.co.uk) that bactosomes™ can be thawed and re-frozen five times with little loss of enzyme activity. A similar result is apparent for the comparison of the

metabolism of diltiazem in bactosomes™ (Figure 3.13) and rat liver microsomes (Figure 3.11).

As was seen with different microsomal concentrations, the apparent metabolic rate, (percentage remaining), can be varied by altering either the drug or the bactosomal concentrations (Figures 3.12 and 3.13). The metabolism of diltiazem (Figure 3.13) indicates that with the higher drug concentrations, the rate of metabolism may not be sufficient to be able to determine the metabolic rate from the background activity (no bactosomes™), and therefore the lower concentrations are more suitable for this purpose. The metabolism of verapamil by comparison (Figure 3.12) is much more readily distinguishable at higher concentrations.

3.6.3 Selective microsomal inhibition

Employing selective microsomal inhibitors can be used to identify the metabolising CYP enzyme by showing a reduction in the rate of metabolism when an inhibitor of a known enzyme is present. Table 3.5 gives the three inhibitors tested in this study. Other potential inhibitors of different CYPs can be found at http://medicine.iupui.edu/flockhart/table.htm.

Inhibitor	Enzyme Inhibited
Furafylline	CYP1A2,
Ketoconazole	CYP2C19, CYP3A4
Sulfaphenazole	CYP2C9

Table 3.5: Selective CYP inhibitors used with the active CYP enzymes.

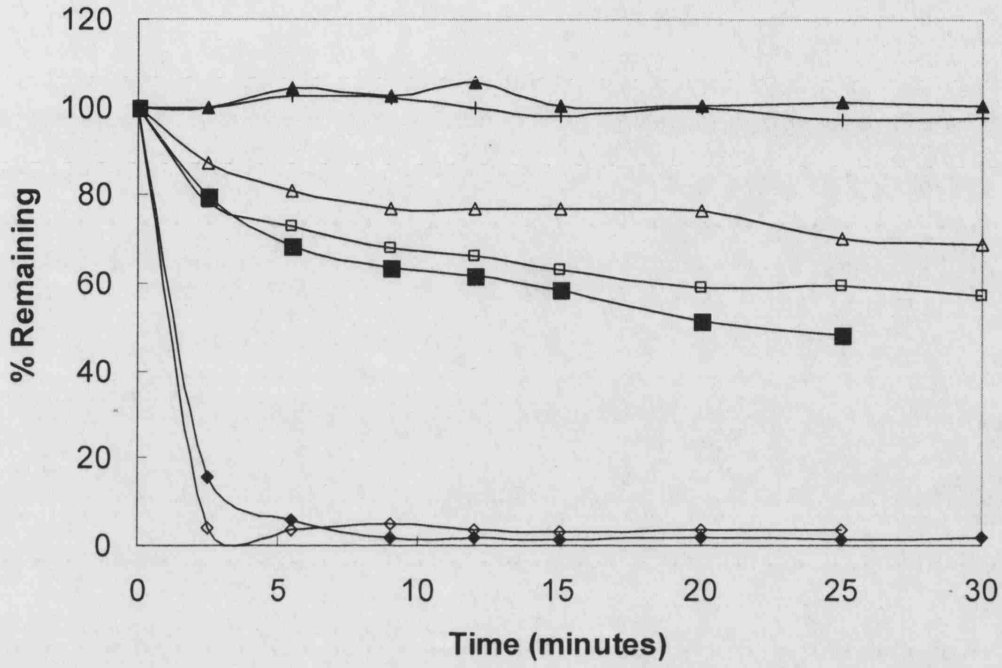


Figure 3.12: Bactosomal metabolism of verapamil. Where:

◆ 3 µM verapamil, 250µL NRS, 25 µL bactosomes™

◇ 3 µM verapamil, 250µL NRS, 50 µL bactosomes™

▲ 3 µM verapamil, 250µL NRS, no bactosomes™

△ 30 µM verapamil, 250µL NRS, 25 µL bactosomes™

■ 30 µM verapamil, 250µL NRS, 50 µL bactosomes™

□ 30 µM verapamil, 500µL NRS, 25 µL bactosomes™

\+ 30 µM verapamil, 250µL NRS, no bactosomes™

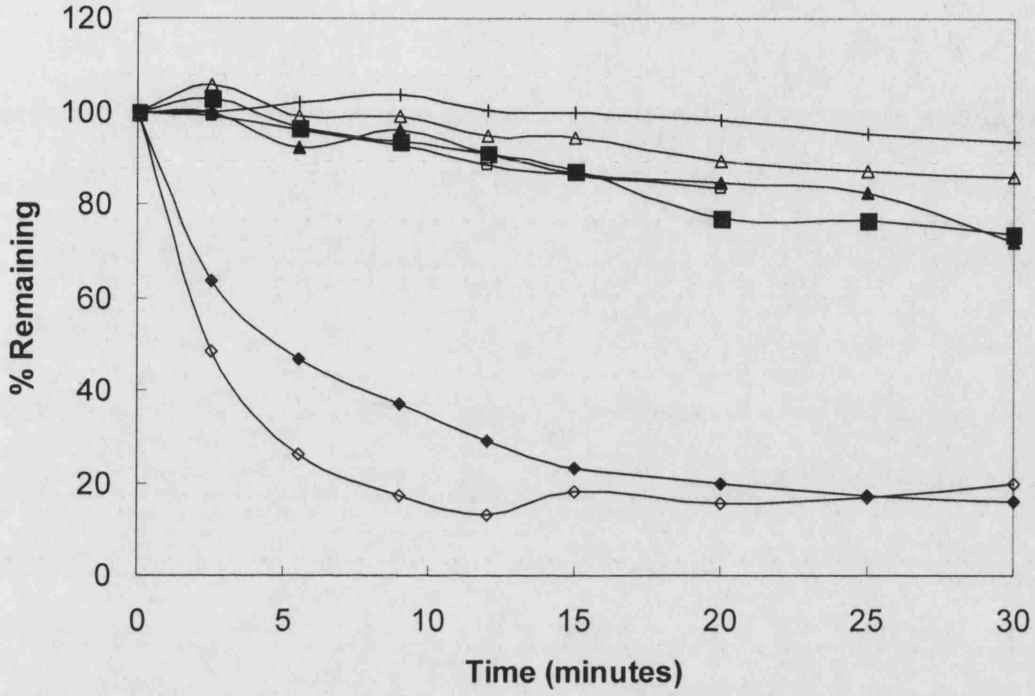


Figure 3.13: Bactosomal metabolism of diltiazem. Where:

◆ 3 μM diltiazem, 250μL NRS, 25 μL bactosomes™

◇ 3 μM diltiazem, 250μL NRS, 50 μL bactosomes™

▲ 3 μM diltiazem, 250μL NRS, no bactosomes™

△ 30 μM diltiazem, 250μL NRS, 25 μL bactosomes™

■ 30 μM diltiazem, 250μL NRS, 50 μL bactosomes™

□ 30 μM diltiazem, 500μL NRS, 25 μL bactosomes™

\+ 30 μM diltiazem, 250μL NRS, no bactosomes™

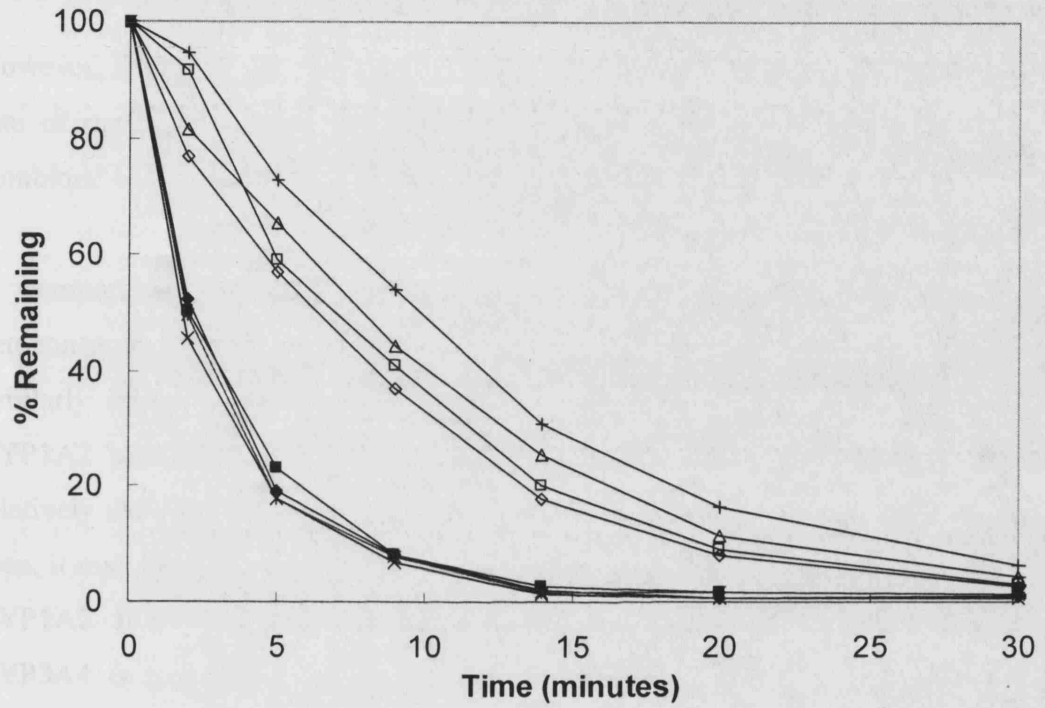


Figure 3.14: Metabolism of verapamil with 10mM of different selective inhibitors.

Where:

◆ furafylline, ◇ ketoconazole, ▲ sulfaphenazole, △ furafylline and ketoconazole

■ furafylline and sulfaphenazole, □ ketoconazole and sulfaphenazole, + furafylline,

sulfaphenazole and ketoconazole, × no inhibitors

As verapamil is metabolised predominantly by CYP3A4 and to a lesser extent CYP1A2, the inhibitors of both of these enzymes would be expected to show a reduction in the rate of metabolism. From Figure 3.14, it appears that of the single inhibitors, only ketoconazole significantly reduces the rate of metabolism. By comparing the different combinations of ketoconazole with another inhibitor, it does appear that furafylline shows greater inhibition than sulfaphenazole,.

However, as the combination of all three inhibitors shows the greatest reduction in the rate of metabolism, it may simply be the case that the presence of an increased combined concentration of inhibitors proves somewhat toxic to the microsomes.

A comparison of the metabolites produced in the absence and presence of ketoconazole (Figure 3.15), shows that the rate of production of all metabolites is similarly inhibited. Therefore it is not possible to draw conclusions of whether CYP1A2 produces only a single metabolite or whether it is responsible for the relatively slow production of a number of metabolites. Due to this similar inhibition seen, it may be the case that it is either norverapamil or D-703 that is metabolised by CYP1A2. It is clear however from this process of inhibiting different CYPs, that CYP3A4 is responsible for a significantly higher proportion of the metabolism of verapamil than CYP1A2.

From their study of the metabolism and mechanism of ten CYP3A4 substrates, Kenworthy *et al.* (1999) found three distinctly different types of inhibition and suggested that multiple probe substrates (at least one from each type of inhibition) be used for the assessment of drug-interactions. Therefore to be effective this method is likely to require the use of multiple different inhibitors for each type of metabolising CYP. However, as CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP2E1, and CYP1A2 are responsible for the oxidative metabolism of more than 90% of drugs (Williams *et al.*, 2004), initial screens inhibiting these enzymes are an attractive option. By using this method of CYP identification an indication of the potential drug-drug interactions could also be predicted.

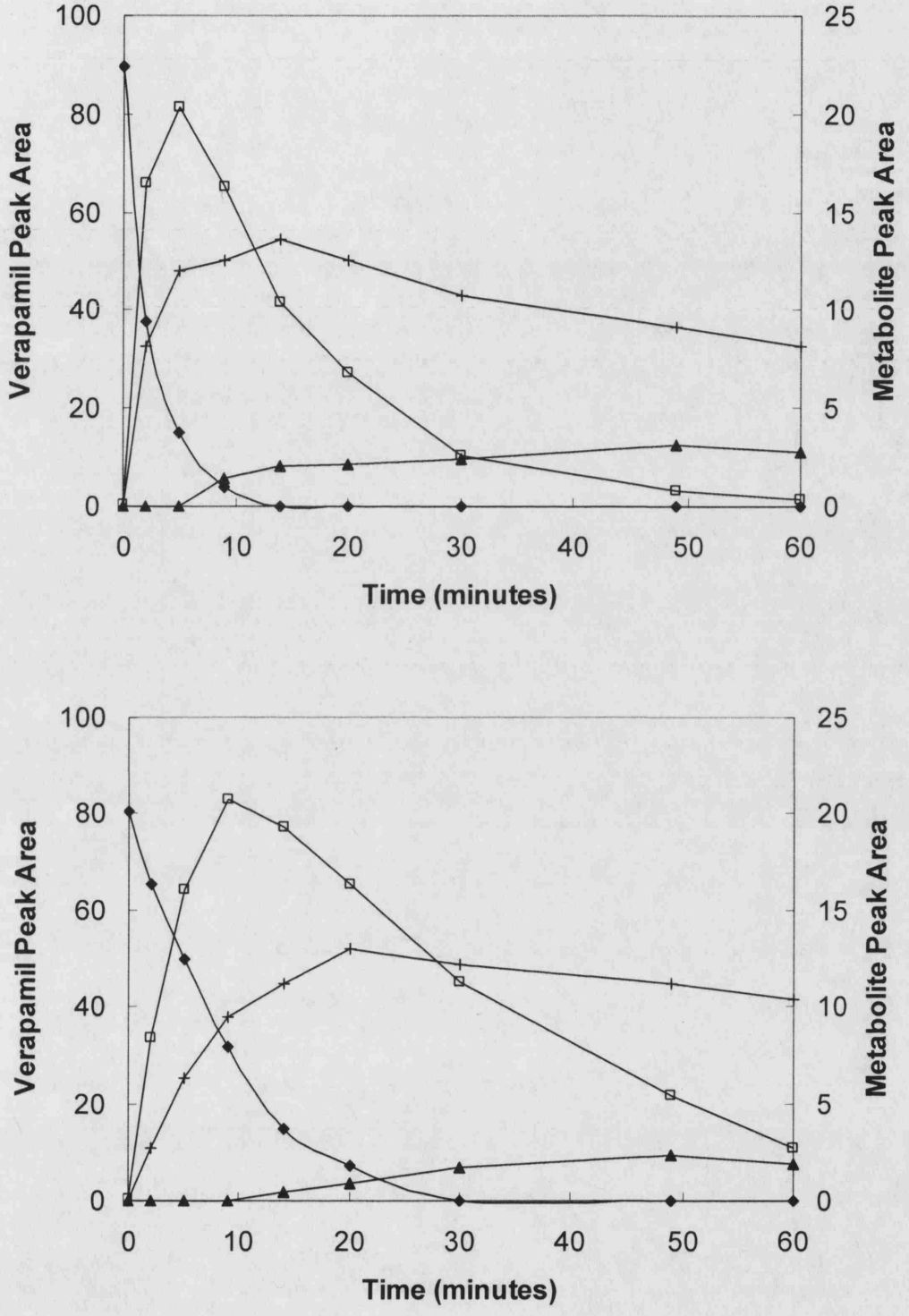


Figure 3.15: Metabolism of verapamil by BD Biosciences rat liver microsomes and fresh *In vitro* technologies NADPH regenerating system in the absence (Top) and presence (Bottom) of the selective CYP3A4 inhibitor ketoconazole. Where:

◆ Verapamil, □ Norverapamil & D-703, + D-617, ▲ D-620

Whilst inhibition of co-administered drugs is the predominant cause of the majority of drug-drug interactions, the knowledge of selective inhibition can also be highly beneficial. For example ketoconazole has been used to inhibit the metabolism of cyclosporin in order to reduce the required dose of this (expensive) drug (Gerntholtz *et al.*, 2004). For similar reasons the drugs paclitaxel, docetaxel and topotecan have been given co-administered with inhibitors (Kruijtzer *et al.*, 2002). By adopting this method of CYP identification, such potential beneficial effects may be found.

3.7 Stage 3: Scale-up of metabolite production

Internet search found that the only commercially available recombinant host cells that express CYPs are wild type organisms. Due to the generic nature of the "CYP" expressed and the low levels of enzyme present, these would not be ideal candidates for the scale-up of metabolite production. A preferable solution would be to hold a bank of cells over-expressing individual CYP enzymes which would facilitate the selective production of individual metabolites.

As no suitable host cell could be found, it was not possible to continue with the scale up of the metabolites of verapamil or diltiazem. However the production of potentially suitable cell hosts and their use in relatively large scale biocatalysis has been shown by several groups (Andrews *et al.*, 2002; Ahn and Yun, 2004; He and Chen, 2005; Omasa *et al.*, 2005; Gamble *et al.*, 2003), as has been discussed in section 1.3.4.3. Such recombinant host cells have been shown to give comparable metabolic rates to those seen in liver microsomal metabolism, yielding the same metabolites at large scale (Yamazaki *et al.*, 2002).

3.8 Conclusions

3.8.1 Metabolite identification and reactant concentrations

The proportional response to changes in microsomal and drug concentrations enables the tailoring of the assay to suit individual compounds. If the microsomal metabolism of a drug can be standardized so that the half-life is approximately constant, then a

much faster method can be set-up. This also means that later experiments in the path can be run with a much lower volume (100μL) with the reaction quenched at the end of ten minutes simultaneously in all wells. This would enable a high degree of automation and a high level of sample throughput that would allow a large volume of inhibitors and bactosomes to be tested simultaneously.

For drugs with shorter half-lives (under five minutes), using higher drug concentrations (50-100μL) or lower microsomal concentrations (25μL or lower) will increase the half-life making results simpler to analyse. For drugs with longer half-lives then the converse is relevant, with the amount of microsomes required increasing with a potentially smaller concentration of drug used to give a shorter half-life. The use of higher concentrations of drugs gives both larger and more identifiable peaks, particularly of metabolites that are produced in small amounts or that give a low LC-MS/MS response. It is noted that when the amount of a drug sample available is small and precious (as is likely the case with newly developed drug candidates), then using lower concentrations of microsomes is likely to represent a cheaper and more viable alternative.

3.8.2 Identification of the CYP responsible for metabolism

3.8.2.1 Panel of liver microsomes

Due to the lack of characterisation of the microsomes available (*In vitro* Technologies provides an even less exhaustive characterisation), the technique of comparing drug metabolism across a panel of microsomes is difficult to perform. Initial results on a comparison of liver microsomes from only two different species appear negative. However, by employing a panel of liver microsomes from a higher number of species, this method may assist in the choice of a species for clinical trial toxicology studies. This technique is also potentially the most efficient method of CYP identification as the complete range of CYPs can be assessed in relatively few experiments.

To be able to function properly, either purchased microsomes need complete accurate CYP quantification or a method of characterising the CYP concentration after

purchase is required. Snawder and Lipscomb (2000) developed an ELISA assay for this purpose that was reportedly robust, however the additional time and cost of performing this additional testing makes this method comparably less attractive.

3.8.2.2 Bactosomes

The method of identifying the CYP responsible for drug metabolism is both very specific and simple to employ. A potential weakness of this method is that another metabolising CYP may be overlooked, leading to key active or toxic metabolites produced by an alternative CYP being neglected. To prevent such an outcome, a complete panel of bactosomes (fourteen are currently available) would be required and the cost and analytical processing requirement of this may prove disadvantageous. As stated in section 3.6.3, only six CYPs are responsible for the metabolism of 90% of drugs and thus employing only six different CYPs does appear more efficient, if not necessarily conclusive.

Due to the ability of bactosomes™ to maintain their activity for a number of freeze-thaw cycles, it would be possible to develop a standard incubation plate, frozen and ready for use. This would facilitate in the rapid testing of multiple drugs and speed up the turn-around time.

3.8.2.3 Selective Inhibitors

The use of selective inhibitors gave a positive identification of the primary metabolising enzyme of verapamil. However, due to the relatively slow rate of metabolism shown by CYP1A2, this enzyme would have been missed as a metabolising enzyme of verapamil. From this it appears that this method of identifying the CYP responsible for drug metabolism should be used with a degree of caution. The potential benefits of gaining an insight into potential of drug-drug interactions and the possibility of co-administering selective inhibitors should not be under-estimated.

It is also noted that whilst the lack of selectivity of inhibitors appears to be a negative result, this could be used to aid the process. Inhibitors could be employed as a pre-screen prior to the use of an additional method of identifying the two or three CYPs potentially responsible for metabolism.

3.8.3 General conclusions

As no suitable cell line could be found for the scale up of metabolite production, no further progress could be made. However the results discussed to date indicate that the following potential steps could be used in the scale-up of metabolite production:

1. Microsomal metabolism of a drug under standard conditions in both human and rat microsomes. The metabolites identified and drug half-life calculated from this can determine the concentration of microsomes, bactosomes and drug solutions for subsequent steps. For drugs with shorter half-lives, smaller concentrations of microsomes and higher drug concentrations should be used for all but the inhibitor studies. For longer half-lives the reverse is true.
2. Employing inhibitors as a pre-screen to identify the range of CYPs that are potentially responsible for metabolism will provide information on the CYP likely to be responsible for metabolism. From this only two or three different bactosomes™ would be required, and the complete range of CYPs could still be tested rather than concentrating on only the 6 CYPs responsibly for the majority of metabolism.
3. Use of one of several pre-formulated plates containing the range of available bactosomes at 100μL scale that correlate with the potential results of the inhibitor pre-screen. The reaction should be quenched by 100μL ACN at a time determined from step 1 above.
4. Use of a combination of the required selective inhibitors in conjunction with bactosomes to allow the selective metabolism of the desired metabolite to the maximum levels possible. This should be a time-course study to yield the time when the metabolite(s) of interest are produced in the greatest quantity.
5. Growth of yeast, insect cells or human lymphoblastoid cells co-expressing the individual CYP enzyme that has been identified as responsible for metabolism

in step 3, with human CYP450 reductase co-expressed to remove the need for NRS solution. This should allow biotransformation of the drug at large scale (dependent upon the concentration of drug required as determined in step 1). The use of selective inhibitors may be required to reduce secondary metabolism to yield the metabolites individually at their maximal concentration.

6. Separation and purification of the metabolite produced using a technique such as preparative HPLC.

Whilst the scale-up of metabolite production should be possible for many drug candidates using the methodology described here, many drugs are neither metabolised by a single CYP, nor indeed by CYPs alone (Tredger and Stoll, 2002) and thus such a single CYP strategy may be ineffective for some drug candidates.

4 Strategy dynamics of a start-up CRO

4.1 Aims of the chapter

To develop a strategy that would enable the successful start-up and optimal subsequent operation of a contract research organisation (CRO) by:

- Developing a model of the system.
- Testing the model's potential for use in scenario planning
- Finding the best strategy

4.2 Introduction

4.2.1 Strategy

"In strategy it is important to see distant things as if they were close and to take a distanced view of close things" Miyamoto Musashi (Kendo Master – 1645).

Strategy became a key business driver in the 1980s after Michael Porter's seminal work on industrial forces, which provided powerful explanations of the competitive environment and its effect on constraining the opportunity for profits (Porter, 1980 & 1985). The success of strategy depends not only on the formal strategic knowledge of a management, but also on a deep and thoughtful understanding of how their firm operates in the industry in which they operate. It is because of the thought that is provoked by applying Porter's models that they still form the foundation of the development of strategy at many of the top firms. A key flaw in such strategic focuses is that they help to explain which strategic resources *currently* make a firm successful and how it can improve profitability in the *current* business environment. In rapidly developing industries it often provides little assistance in creating competitive advantage into the *future* (Fowler et al., 2000).

The rapidly developing social, economic, political and technical environment of the last few decades has meant that the largely static results of formal strategy techniques

are less reliably predictive. For this reason the use of formal strategy tools has been in steady decline for many years (Bain & Co., 2005). A recent review of available strategy tools (Huyett and Roxburgh, 2000) led to the conclusion that little advancement on Porter's theories has been made on *where* to compete. On *how* to complete some general principles such as "build and sustain strategic resources", "concentrate on core competencies" were recommended.

To lessen the problem of rapidly developing environments, an emergent strategy process has been developed (Figure 4.1). Whereas traditional approaches analyse the strategic options, such as selecting the best at the time and then implementing it, the emergent approach is a continual process of analysing options and developing suitable strategies. The strength of this approach is determined by the amount of time and effort that is spent in continually developing and implementing strategies.

The resources based view of a firm widely acknowledges that capabilities are unique and important for achieving a sustained competitive advantage (Wernerfelt, 1984; Peteraf, 1993; Shapiro, 1999 Coates and McDermott, 2002). However Barnett and Brugelman (1996) recognised that a dynamic model is required to assess future trends rather than to concentrate on historical evidence.

Strategy Dynamics combines the emergent approach with the Resource Based View enabling strategic resources to be modelled over time. The complex and intertwined nature of strategic resources is often neglected (Coates and McDermott, 2002), but it is the mathematical interrelation of these resources which allows different strategies and scenarios to be simulated *in silico* rather than having to adopt a trial and error based on instinct.

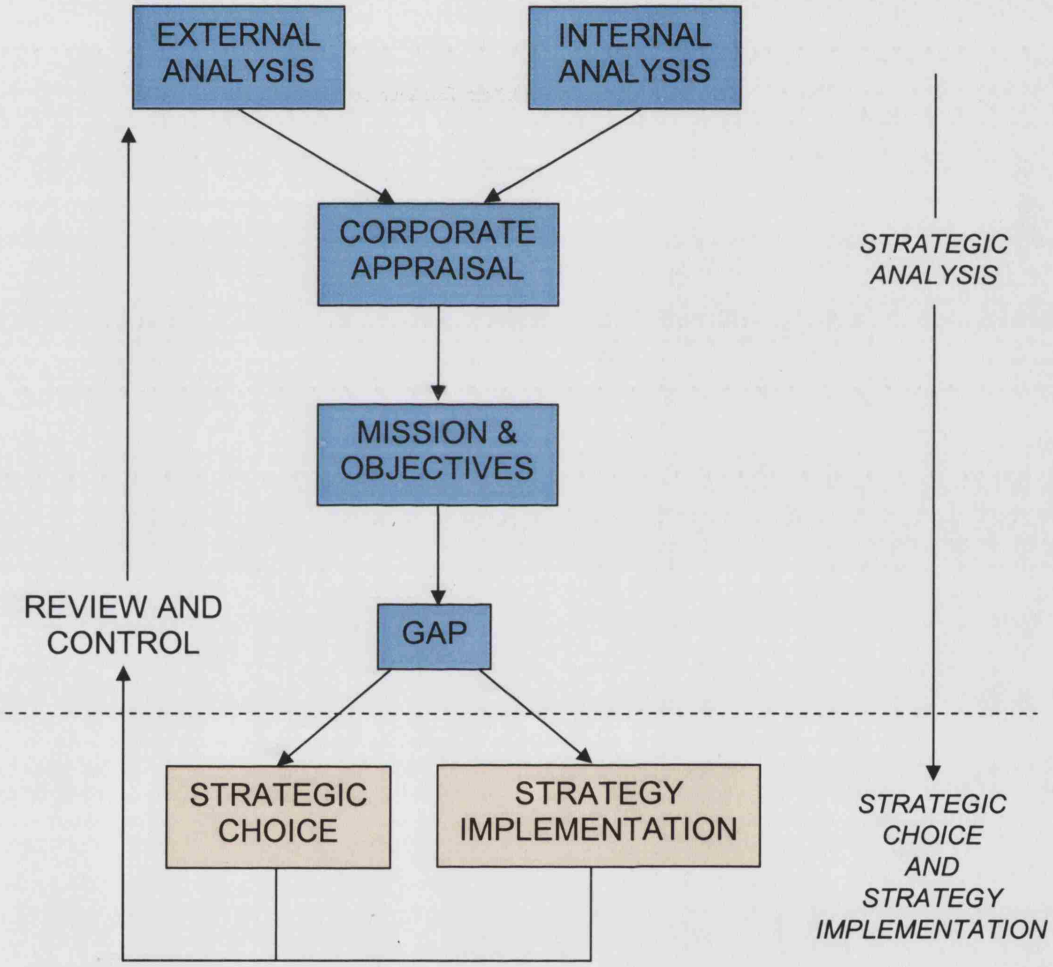


Figure 4.1: The emergent strategy approach. Adapted from the ICAEW Business Management Tuition Guide, 2004/5.

Whilst strategy may appear to be unconnected to biochemical engineering, the dynamic resource based modelling approach has similarities to metabolic pathway modelling (Carlson *et al.*, 2002; Klamt and Stelling, 2003; Carlson and Srienc, 2004). The actual part played in each metabolic pathway is defined by nature, but can be difficult to model due to the complexity of measuring individual pathways. In comparison the dynamic interdependent relationships of strategic resources are defined by the market and the state of scientific research, but due to the high number of variables, are also difficult to model.

The development of both models requires a great deal of measurement and evaluation, be that of metabolic reaction kinetics or customer feedback on service quality, and then subsequent improvement. That being said, the benefits allow for a much greater understanding, whether it is of the cell function or business environment.

4.2.2 Contract research organisations

Heffner (2004) noted that by 2004, 42% of pharmaceutical drug development expenditure was spent on outsourcing, compared to only 4% in the early 1990s. Bain & Co (2005) found that executives are "outsourcing like crazy" to try to reduce working capital with 75% of people admitting to using this management tool. Deloitte Consulting (Kager and Dettmar, 2000) interviewed twenty two executives at thirteen leading pharmaceutical companies and found that they outsource if there is reluctance to invest in technology which:

- Is new to the world and represents significant risk of failure.
- Is new to the company and represents risk from poor integration with existing processes and people, as well as the expense of developing it.
- Is not a competency in which the company wants to develop an expertise.
- Despite being a valued technology currently, the company is uncertain just how long the technology might remain useful.

One factor that necessitates sound strategic decisions is the nature of the variable business opportunities for CROs, with often little knowledge of whether there will be

repeat custom and thus the available funds to further invest in new technology and growth (Madley, 2004). Kager and Dettmar (2000) report that outsourcing is changing to become a strategic long-term partnership rather than a short-term tactical arrangement and therefore larger contracts and preferred vendors status will increase the level of stability and increase the ability to be able to predict future growth potential.

As product life cycles shorten and competition becomes increasingly innovation based, increasing attention has been given to how firms generate competitive advantage (Teece *et al.*, 1997). Generally successful products in rapidly developing industries provide only short-lived competitive advantage.

Due to the rapidly changing scientific environment of contract research organisations, where researchers and scientific experts become business managers, the approach to strategy is often one of scepticism. Business decisions tend to be reactive rather than governed by strategy. Furthermore they are made on either instinct, trial and error or follow a more general business strategy such as cautious growth. By the nature of the environment such decisions often prove disastrous, as is shown by the high rate of business failure among scientific start-up companies and the relative difficulty in finding venture capital funding. Where start-ups are in a competitive environment, the importance of key business decisions on how to grow, where to invest the limited available capital and in which areas to focus are crucial. Additionally, whilst large companies may be able to develop multiple competencies, smaller companies may need to focus on one or two (Fowler *et al.*, 2000). It is for these reasons that strategy dynamics represents a good potential solution to a complex problem.

4.3 Model development

The principle of strategy dynamics is derived from engineering's control theory, where each resource can be considered as a tank into which the resource can be added to or can drain away. The principle is simply illustrated in Figure 4.2, using staff numbers as the resource, recruitment as the method of adding resource and redundancy and natural wastage as methods the resource drains away. Unlike traditional resource based approaches, Strategy Dynamics treats intangible and uncontrolled factors such as clients and quality of research as a resource. The control over resources such as number of clients is driven by input and output flows. These flows are themselves driven by other variables, for example advertising, price, reputation and quality of previous work performed effect the number of clients from whom work is won.

Warren (1999a) introduces the principles of strategy dynamics which has been further developed with a focus on rivalry (Warren, 1999b) and intangibles (Warren 2000), but briefly they take the following steps:

- Definition of the time-path of the strategic challenge facing the firm.
- Identification and definition of the strategic resources that must be developed, defended and connected.
- Selection of the three or four core resources that must be built and defined; generally at least one associated with supply (i.e. staff) and one associated with demand (i.e. customers).
- For each core resource specification and measurement of the inflow and outflows, collecting a recent history on each.
- Identification for each the other resources which drive or constrain the losses and gains.
- Combining these into a composite resource map.
- Adding time-charts for as many items on the resource map as possible.

Identifying the key management handles (decision levers) in the system.

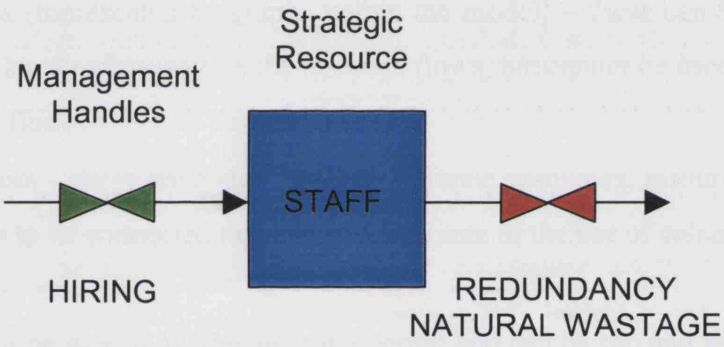


Figure 4.2: Basic principle of strategy dynamics, illustrated using staff numbers.

Further guidance on model construction and the strategy dynamics methodology can be found in the recently published book written by Warren (2002).

The model was developed using MyStrategy™ (www.strategydynamics.com). This software enables the development of a model of any system or company by allowing users to add:

- Individual strategic resources (represented by square boxes within the model) – these are increased or decreased within the model by resource flows.
- Resource flows, described by Warren (2002) as Management Handles (represented by circles within the model) – these can be set to different absolute values within each month or year, or can be calculated as based upon any formula and adjusted by any variable or strategic resource.
- Variables (represented by graphs within the model) – these can be calculated in a similar, flexible way, as the resource flows, but cannot be used as the main resource flow.
- Connectors – these are added to allow different resources, resource flows and variables to be connected together and facilitate in the use of calculations.

The software can be used to model any time period and can be run and stopped at any time within the period modelled to allow assessment of how the different resources interact with each other.

4.4 Key resources and model development

As has already been stated, strategy dynamics takes a resource based view where key resources and their interactions are modelled over time. Generally the measurement of competencies, which are often intangible and dynamic, is difficult (Fowler *et al.*, 2000). However, by producing empirical models based on real data or projected data, mathematical links between the key resources which lead to competencies can be modelled.

Whilst a static resource based view approach to strategy concentrates on tangible resources (Physical, human, financial, information technology, marketing,

organizational and legal resources – Mahoney and Pandian, 1992) it tends to ignore the often equally important intangible resources and resources not in the control of the company i.e. customers. Strategy dynamics by comparison enables such difficult to measure intangible factors and uncontrolled tangible resources to be modelled and via linked business responses, measured and then refined over time. Warren (2000) concluded that business performance could not be effectively modelled unless such intangible resources are also rigorously dealt with. Carmeli (2001) studied only the effects of intangible resources and found a large differential between high and low-performing firms. Figure 4.3 describes the key resources and features of the strategy dynamics approach in terms of their focus and how tangible they are.

A key strategic resource must meet the following criteria (Coates and McDermott, 2002):

- It is difficult to imitate.
- There is asymmetry among the firms with respect to ownership.
- It must provide opportunities for the firm.

The strategic resources of a CRO are proposed in Table 4.1. These are later described in more detail.

Traditional resources	Other resources	Soft factors
Number of assays available	*Customers*	*Service quality*
Analytical equipment time	Knowledge and expertise	Research staff experience
Research staff time		
Capital		

Table 4.1: The strategic resources of a contract research organisation. The key strategic resources identified are shown in italics.

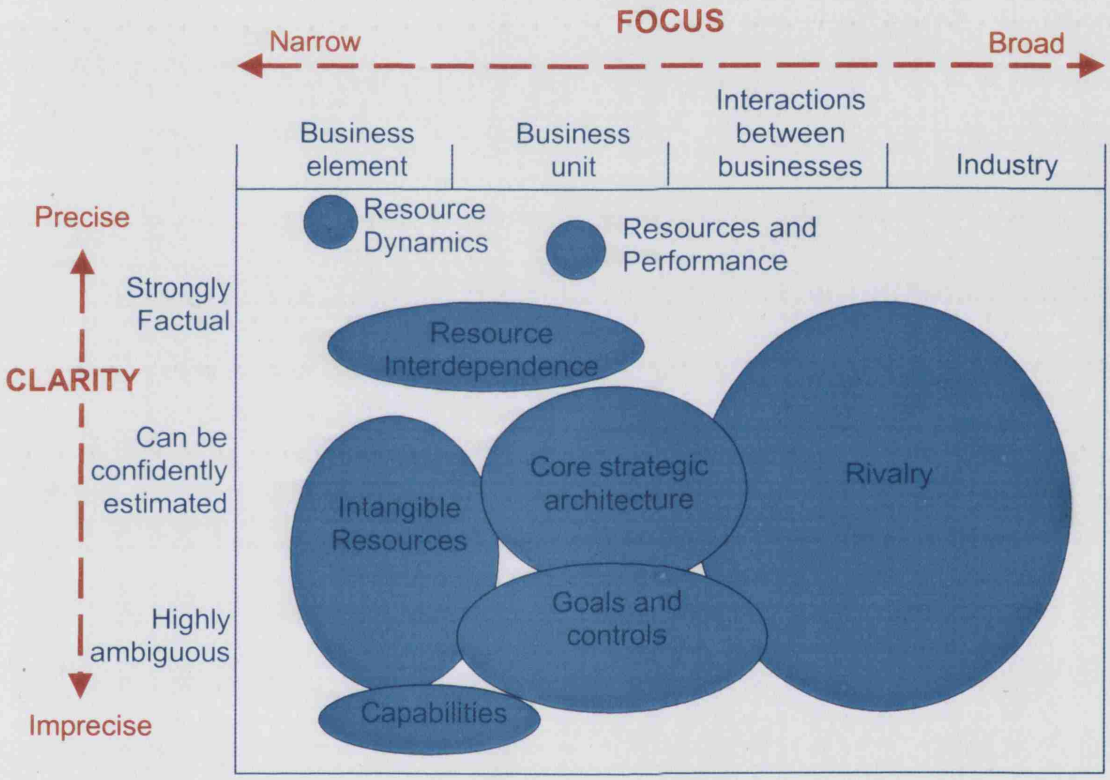


Figure 4.3: How the various components of strategy dynamics range in terms of focus and clarity. Adapted from Warren, 2002.

Whilst the model was developed as a complete resource map, for ease of understanding this has been displayed and discussed in sections (Figures 4.4 to 4.10) to improve clarity.

The model was developed based upon actual data from start-up data for a new ADMET (Absorption Distribution Metabolism Excretion Toxicology) department in which potential drug candidates are tested for their suitability for human used based upon a wide variety of experiments, the results of which are predominantly analysed using mass spectometry. The company modelled, Evotec OAI, is a contract chemistry and biology research organisation in which the ADMET department is a small part.

For this study, a 10 year period was selected as the most relevant, due to number of potential unknown factors in the future, such as the potential collapse of funding within the industry and demand for outsourcing of research. The first 2 years of data simulated within the model are based upon actual department performance (2002-2004), with the remaining 8 years being predictive of performance (2004-2012). Estimates of resources, such as the potential number of assays available for development from public sector research, the number of potential customers and others were all made by the head of the ADMET department of Evotec OAI, Dr Caroline Ward, and were corroborated where possible by literature research and internet searches.

There is no skeletal strategic architecture provided within the MyStrategy^{TM} software and this architecture has been developed in its entirety, specifically for this project. The structure of the model has been reviewed by Professor Kim Warren of London Business School and the accuracy of the model was tested by benchmarking against the first two years of operation of the department.

Due to the commercial sensitivity of the data, financial performance figures have been erased from the model (Figure 4.10) and where requested due to commercial sensitivity data has not been disclosed.

The predictive power of the model is utilised by analysing the key resource constraints over the predictive period and is also used to assess the impact of a series of scenarios in Section 4.6. For the discussion of model development that follows in the remainder of Sections 4.4 and in Section 4.5, model screenshots taken are based upon a scenario of maximum growth. The rationale behind this scenario is further described in Section 4.6.2.

The simulation can be stopped at any time to assess and modify the strategic resources and the time frame studied can be set as required. it is by this method of analysing resource limitations at any time, that a strategy of maximum business growth was developed.

4.4.1 Management handles

The key strategic resources have been described above. To be of use, the simulation must be able to allow management to make decisions about the future. Whilst any of the variables and equations used in the model can be modified, there are a number of key management handles which can be adjusted to modify the inter-related flow of resources. These have been ringed red in the relevant Appendix VI figures:

- Research staff recruitment and the experience of new hires (Figure 4.5)
- Analytical equipment purchase (Figure 4.4)
- Mixture of time spent on researching new assays versus that spent performing client assays (Figure 4.5).
- Concentration of research on public domain versus in-house developments (case by case analysis required for optimum decision – possible in-house developments conducted first in this simulation)
- Mark up charged on assays (Figures 4.9 & 4.10)

Each management handle will be discussed in turn in the relevant section.

4.4.2 Analytical equipment time

-Most chemical and biochemical reactions are followed using high-throughput analysis, such as Mass Spectrophotometry (MS) and High Performance Liquid Chromatography (HPLC). With the advent of high throughput screening techniques using ninety six well plates, the time available on these expensive machines can become resource limiting, with an increase in analytical time representing a significant investment where returns often need to be assured prior to purchase. Additionally with rapidly increasing technological advances in such equipment and therefore an increased risk of obsolescence, decisions on when to purchase such equipment can in itself justify the need for detailed strategic analysis.

4.4.2.1 Assumptions

In the development of this section of the model (Figure 4.4), the following assumptions were used:

Resources

- Analytical Equipment Days – It has been assumed that each analytical instrument (i.e. MS, HPLC) would run for 300 days per year, regardless of the equipment type, the remainder of time being lost through breakdown or used for maintenance.

Flows

- Purchase of New Equipment **<Management Handle>** – When analytical equipment is being used at full capacity, management can chose when the demand is sufficient to purchase a new analytical instrument. In reality the demand for new equipment is also driven by the need for additional capability, to enable to performance of new assays being developed. Similarly new equipment is potentially likely to increase the throughput of analysis possible. However, for simplicity it has been assumed in the model that equipment is

purchased solely based upon capacity requirements, with any additional capability requirements driving instead the choice of equipment purchased.

- Redundancy – Whilst in reality equipment is likely to become redundant either through obsolescence or through breakdown. Given that this would have no net impact on the ability of the CRO to win clients or perform work, for the simplicity of the model, it has been assumed that no such redundancy will result. If this were to occur, an additional analytical instrument would be purchased, with no other effect than to have an increase in the cost in the year of purchase.
- Client Assays Initiated – As this flow is impacted my more factors than the Client Analysis Time available (see figure 4.6), it is described in Section 4.4.4.1.

Variables

- Analysis Time on Assay Development – It has been assumed that 5 days of analysis time will be required to develop external assays, i.e. those published in journals, with 10 days required to developing new assays based on internal expertise.
- Client Analysis Time – It has been assumed that the CRO has the staff, expertise, money and confidence to seek rapid growth. Therefore it has been assumed that assays are developed in preference to performing client assays, however due to the high availability of potential externally developed assays upon start-up, the ratio of time spent between assay development and client work is controlled separately, as shown in figure 4.5 and discussed 4.4.3. The reality of needing to gain experience in performing assays in order to be able to develop further assays effectively and to win customers early is modelled separately and is discussed within Section 4.4.7.
- Analysis Time Remaining – This is a calculation of the Analytical Equipment Days available compared with those used on both Client Assays and in Assay Development.
- Analytical Efficiency – Estimates of the number of Analytical Equipment days available and required, used in the assumptions above were made by the head

of research in the department modelled, based upon her experience. The model did not reflect the reality of how the department had actually performed over the initial two years of operation. Therefore the variable of Analytical Efficiency was introduced to reduce the level of analytical time available to that used. One noticeable reason for this was for the higher level of MS breakdown and servicing required compared to that expected.

4.4.2.2 Model Performance

The modelling of analytical equipment time and its effect on the time available to perform the analysis of client assays subsequent to performing analysis on assay development is shown in Figure 4.4. It can be seen from the model and interpreted from the results shown, that to enable maximum growth three new analytical instruments would be required in 2006, 2008 and 2010 to prevent this resource from becoming growth constraining.

4.4.3 Research staff time

In a start up company the number of possible appropriately qualified researchers is often highly limited. Whilst grants for capital expenditure such as analytical equipment are available, money to pay salaries and for consumables is often in short supply. The ability and knowledge of staff is likely to be limited to a finite number of analytical techniques, and whilst staff can be trained, this takes time.

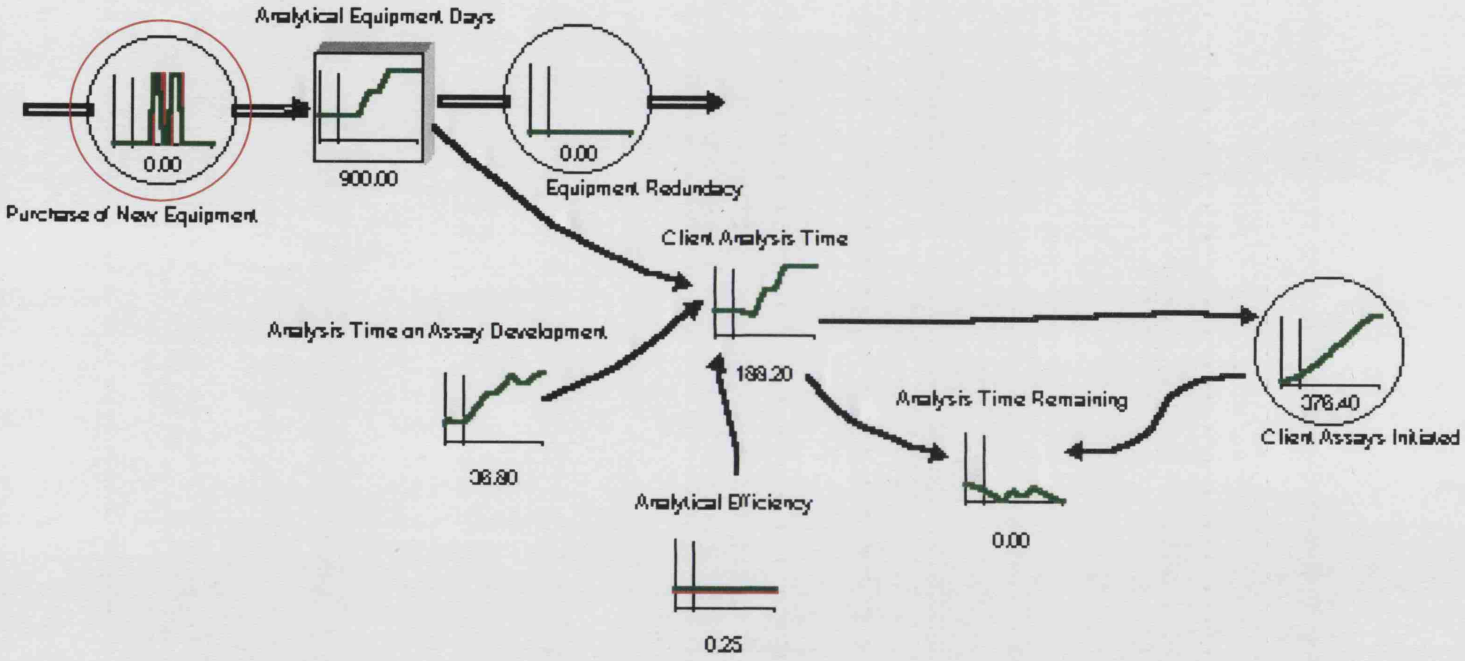


Figure 4.4: Analytical equipment time (days) where the red ring represents a key management handle. Boxes represent key resources, circles represent flows of increasing and reducing resource as indicated by direction of flow, and standard graphs represent variables affecting the speed of flow of resource. The number shown represents the final Y-axis value, the X-axis the years 2002 to 2012, being based on actual results to the left and model predictions to the right of the Y-axis (2004).

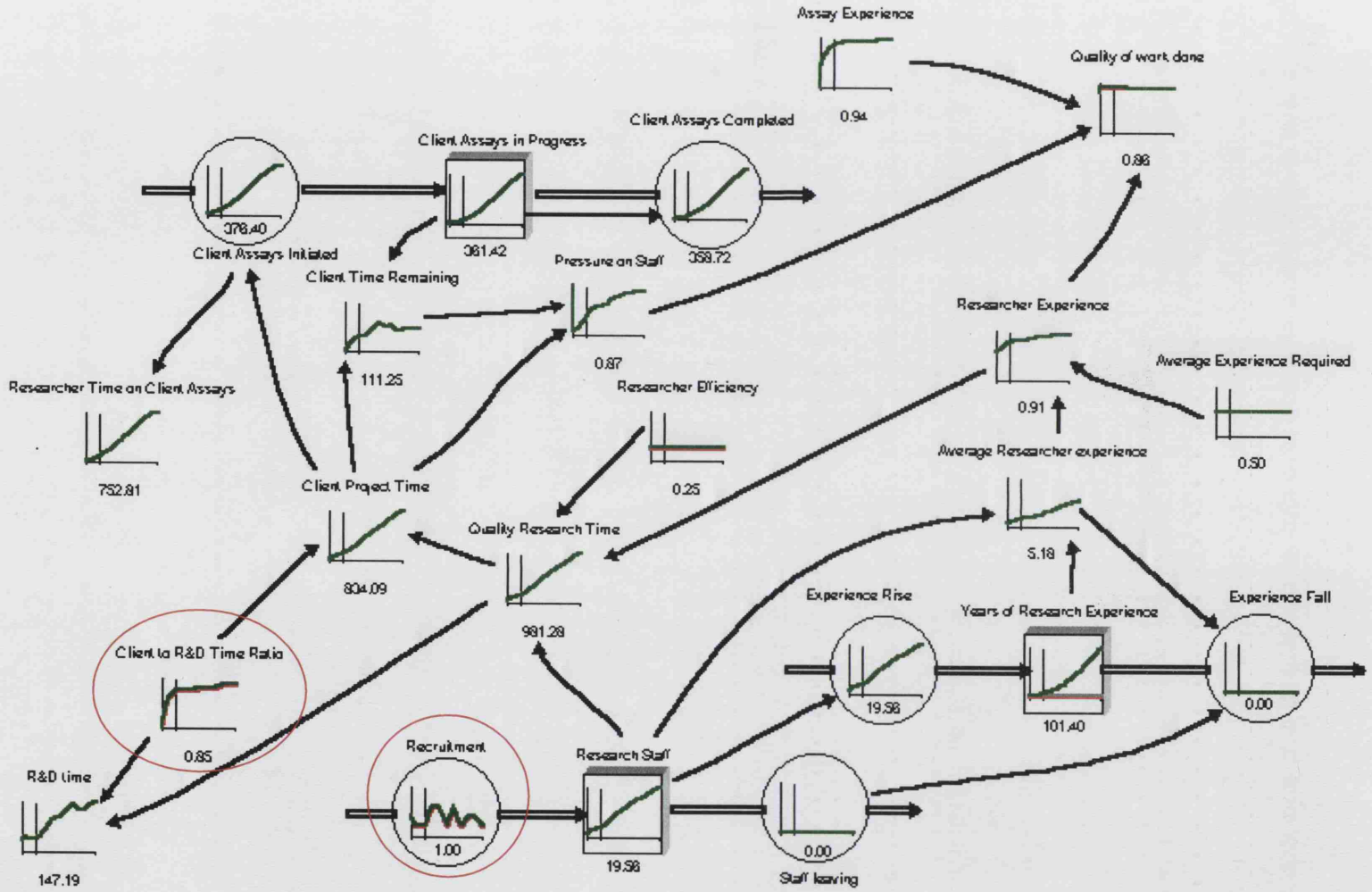


Figure 4.5: Research staff time where key management handles are represented by a red ring.

4.4.3.1 Assumptions

In the development of this section of the model (Figure 4.5), the following assumptions were used:

Resources

- Research Staff – In line with the department created on which this model was based, it has been assumed that the two initial researchers are the primary holders of expertise and subsequent researchers are competent scientists, who must be trained and will become more experienced in the specific assays over time.
- Years of Research Experience – To enable modelling of the importance of the experience of staff, this has been built into the model.
- Client Assays in Progress – Due to the additional inputs into the Client Assays Initiated, as shown in Figure 4.6, this is discussed further in Section 4.4.4.

Flows

- Recruitment **<Management Handle>** – In this scenario of maximum growth shown, it was assumed that sufficient staff (of a suitable experience) were recruited to prevent researcher time becoming the constraining resource. It is assumed that where experience with additional analytical techniques is required to enable effective research into new assays, that suitable staff are recruited accordingly. Suitably qualified scientific staff often take time to recruit, so the ability to predict when they will be required, as facilitated by this modelling, is beneficial in that it allows the effective comparison of early versus late recruitment on the likely prosperity of the business in both current and future years.
- Staff Leaving – Similar to Analytical Equipment Redundancy, it was assumed that no staff will leave the department. Whilst this is likely to be over simplistic, the only result would be for a replacement being hired with a similar amount of experience, with no further impact on the model. The model could be used further to assess the impact of key members of staff leaving the

department, with the resulting lack of experience to develop and perform assays effectively.

- Experience Rise – Each year that a staff member stays within the department, a year of further experience is gained. For simplicity it has been assumed within the model that staff are recruited with limited experience in this field of research. If more experienced staff members were recruited, then this additional experience could be modelled here.
- Experience Fall (/Average researcher experience) – It has been assumed that when a staff member leaves, that they leave with the average level of experience. Whilst this may be over-simplistic, if a scenario modelling the effects of a key member of staff leaving were required, the model could be easily adopted to allow assessment of the impact of this and allow for adequate succession planning.
- Client Assays Initiated/Client Assays Completed – see Section 4.4 and Figure 4.6 for assumptions made.

Variables

- Average Experience Required/Researcher Experience – It has been assumed that to be effective the average researcher experience must be higher half a year. The higher the percentage Researcher Experience, the higher the resultant quality of work and the more "Quality Research Time" available for performing assays.
- Researcher Efficiency – As with Analytical Efficiency (see Section 4.4.2.1), this variable has been inserted to match the amount of research time available, to the amount of research that was actually performed during the first two years of operation. The low value of 25% used reflects the amount of time spent on other training, attending meetings, and other jobs such re-ordering stock and dealing with Clients.
- Quality Research Time – This is calculated based upon the number of Research Staff multiplied by 220 working days, % Researcher Experience and % Researcher Efficiency. Building in the assumption that more experienced staff will be able to work more effectively.

- Client to R&D Ratio **<Management Handle>** – This was set based upon estimated historical levels of time spent between R&D and performing Client Assays in the first two years of operation – 0% at the start of year one, rising to 60% at the end of the initial year, and rising to 75% at the end of year two. The optimal ratio level thereafter was calculated based upon optimisation of the model, as described in Section 4.4.3.2 below.
- Client Project Time/R&D Time – This is calculated from the Client to R&D Ratio as the proportion of Quality Research Time that was dedicated to conducting assays for clients and conducting R&D respectively.
- Client Time Remaining/ Pressure on Staff – Client Time Remaining is calculated as the amount of time that has been made available for Client work, but which is not being used due to a lack of demand. The proportion of client time which is used on performing client analysis has then been simulated as the Pressure on Staff.
- Researcher Time on Client Analysis – It has been estimated that the average Client assay will take 2 days to complete, regardless of the type of Assay completed. Therefore the Researcher Time on Client Analysis is calculated as twice the number of Client Assays Initiated.
- Assay Experience – see Section 4.4.5 and Figure 4.8 for assumptions made.
- Quality of Work Done – As a simplifying assumption, it has been assumed that Quality of Work Done is independent upon the type of assay performed and is calculated as an average quality factor over all assays performed in any year. In the model it has been estimated that given the company standard experimentation protocols and reporting standards that the clients would perceive an 80% quality factor as a baseline, with a further 10% based upon the Assay Experience and Researcher Experience and 10% based upon the pressure on staff. As a critical factor on reputation of the department and the amount of repeat custom, the accuracy of this intangible variable needs to be tracked and remodelled as appropriate based upon findings such as customer satisfaction surveys, as described in Section 5.5.

4.4.3.2 Model Performance

The research staff's time, their experience, utilisation and the combined effect of these on the amount of quality research time available and the subsequent number of client assays they can perform and the number of assays they are able to develop is shown in Figure 4.5.

By analysing the effect of R&D to Client Time Ratio *in silico*, it was possible to optimise whether staff would be best directed towards spending time on developing the number of assays available to customers, or on building a strong customer base. As Customers are a key resources, which are only indirectly controlled by offering a good quality service at a reasonable price, it was found through the model that securing a strong initial customer base is critical for long term success. This is described further in Sections 4.4.5 and 4.7.1. To create the maximum revenue over the 10 year period studied, 80% increasing to 85% of time was found to be best spent on performing client assays and building up the client base.

4.4.4 Client Assays in Progress

The different sources of customers (new, won from rivals and repeat), the number of assays that they request as part of the service (dependent upon the number of assays available and the quality of service) and the number of assays in progress is shown in Figure 4.6.

4.4.4.1 Assumptions

In the development of this section of the model (Figure 4.6), the following assumptions were used:

Resources

Client Assays in Progress – A key resource in the generation of revenue is the amount of work in progress. This is driven purely by the number of Client Assays Initiated.
Potential Assays from Public Domain – See Section 4.4.6, Figure 4.8.

Flows

- Client Assays Initiated – The number of Client Assays Initiated is calculated as the number of customers multiplied by the Number of Assays per client. If maximum growth is not sought then this can be constrained by the time available on the analytical instruments (Client Analysis Time) and the researcher time available (Client Project Time). It has been assumed that each assay will take 2 days of researcher time and ½ day of analytical equipment time to complete. See Section 4.4.5 for the assumptions underlying the number of customers.
- Client Assays Completed – It has been assumed that work for customers takes an average of 3 months to complete, therefore a delay of a quarter of a year has been used to calculate the amount of Client Assays Completed. Whilst this appears a long amount of time, it was based on the average actual performance on the completion of assays in the first 2 years of operation of the department modelled. It is feasible that the turnaround of customer work would improve after this period, as the number of researchers available could work together to

improve efficiency, rather than working on their own assays as has been assumed in the model. This flow should be tracked going forwards, as described in Section 5.5, and the model adjusted to a more reasonable assumption as required.

- New Customers/Customers won from rivals/Repeat Customers – See Section 4.4.5 and Figure 4.7 for assumptions made.

Variables

- Assays available to customers – See Section 4.4.6 and Figure 4.8.
- Client Analysis Time – See Section 4.4.2.1 and Figure 4.4.
- Client Project Time – See Section 4.4.3.1 and Figure 4.5.
- Number of assays per client – Based on historical actual data, it has been estimated that repeat customers will required twice as many assays completing as either new customers or those won from rivals. It has also been assumed that the higher the number of assays developed and made available for customers to purchase, the higher the number that they will request. These assumptions have been bounded to provide the estimated expected range of an average of between 1.2 (historic actual) and 4 assays per customer.

4.4.4.2 Model Performance

Figure 4.6 shows that the number of assays developed increases to 40, and the number of customers increases to over 100 a year by 2012. In the tenth year of operation, it has been predicted that 360 assays will be completed.

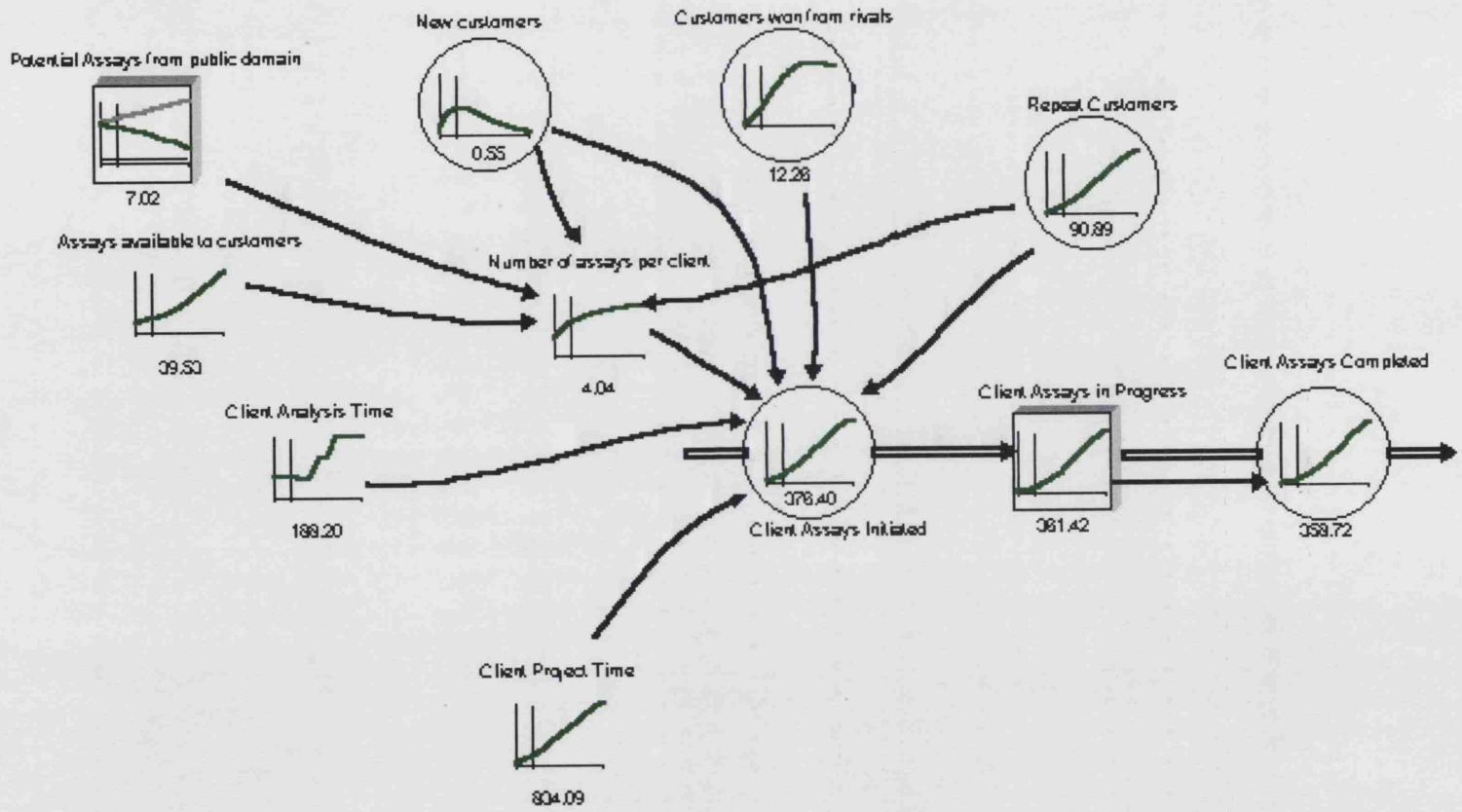


Figure 4.6: Client Assays in Progress.

4.4.5 Customers

As potentially uncertain customer demand is a primary reason for the cautious approach to investment often seen, it is crucial for new CROs to research their potential markets. Generally customers specific to start-up CROs (pharmaceutical, biotechnology and chemical companies) are relatively very large and retain much of the power. Due to their size they are also limited in number and so a key relationship between quality of service and repeat custom has been integrated into the model. The importance of knowing the needs of customers was highlighted by a 2005 survey of executives (Bain & Co., 2005), who found that two thirds of respondents agreed with the statement 'insufficient customer insight is hurting our performance'.

4.4.5.1 Assumptions

In the development of this section of the model (Figure 4.7), the following assumptions were used:

Resources

- Potential Customers – It has been assumed that in the period between 2002 and 2012 there will be a total of 500 potential pharmaceutical, chemical or biological companies who are potentially interested in the type of assays being offered.
- Potential Assay Customers – These are the potential customers which may decide to use this type of assay, either outsourcing to the department modelled, to a rival or developing the capability in-house.
- Customers / Rivals' Customers / In-house Assay Groups – These are the potential ways in which potential customers could decide to adopt these assay types.
- Past Customers – Once customers have been serviced, they become Past Customers, who, dependent upon the quality of the work and it's price competitiveness could become either repeat customers, could decide to use a

rival's service, could develop in-house capability or could decide not to use the assay type again.

- No Longer Using Assays – This is the proportion of the 500 initial potential customers who have decided that they would no longer wish to use these types of assay.

Key assumptions are that it is easier to get repeat customers (if quality is high) than it is to get new customers, which are themselves easier to obtain than customers currently serviced by rivals. Similarly repeat customers are likely to request a higher number of assays than new customers who are likely to be more concerned with the quality and usefulness of the results.

Flows

- Adopters of Assay Type – Driven by the % Awareness of the Assay type and the % Reputation of Assays in Industry, it has been assumed that with aggressive take up of the assay type up to 20% of the Potential Customers will become Potential Assay Customers by deciding to assess the effectiveness of this type of assays.
- Client's In-house Dev 1 – It has been assumed that only 2% of Potential Assays Customers would decide to set-up in-house capability without first attempted to outsource.
- New Customers – The amount of new customers won is calculated as the % Customer Win Potential multiplied by the Potential Assay Customers. Calculation of Customer Win Potential is discussed below.
- Work Completed – This is calculated as the number of Client Assays initiated, divided by the Number of assays per client.
- Lost to Rivals – This has been calculated as the remaining Potential Assays Customers won, i.e. after those that have decided to use this department or set-up an in-house capability.
- Repeat Customers – It has been assumed that these are won through having a high % Quality and Competitiveness, both in terms of cost and quality of service. The maximum proportion of repeat customers being won being 50%,

customers requesting further work 3 months after their last job was completed. This gives a profile similar to that historically observed, equating to the 10 repeat customers in 2004 (i.e. 50% of business coming from repeat customers, 50% from new customers at this time). Due to the promising outlook for the assay type and the high level of repeat custom in the pipeline, it has been assumed that this % repeat customer will continue to 2012.

- Customers won from rivals – It has been assumed that customers using a rival service will be loyal to them and thus twice as hard to win as New Customers.
- Client's In-house Dev 2 & 3 – It has been assumed that Client's are twice as likely (i.e. 4% of clients who have previously outsourced) to set up in-house capability once they have found the outsourced experiments to be useful.
- Assay Leavers 1 & 2 – It has been assumed that 2% of past customers (both own and of rivals) will decide not to use the assay type again.

Variables

- Reputation of Assays in Industry – It has been assumed that the reputation of assays in industry will steadily increase over the 10 years modelled from 40 to 60%.
- Awareness of Assay Type – Awareness of the assay type was assumed to have initially been only 60% in 2002, rising to 75% in 2004 and 99% in 2010.
- New Client % - This is calculated to indicate the importance of repeat custom and calculates the percentage of customers who are new to the department compared to those which are repeat customers.
- Awareness of Company Assays – This has been modelled based upon perceived awareness of the Assays available. Based upon anecdotal evidence, some customers noted that awareness of the Assays available was not well marketed, either through the company website or marketing material. In 2004 it was believed that only around 15% of Potential Assay Customers were aware of the services available. After a marketing campaign planned for 2005, it was estimated that this might rise to 50%, with a subsequent rise to 70% by 2009, from where it was not predicted that this would increase.
- Quality and Price Competitiveness – This is described in Section 4.5.

- Customer Win Potential – This is based upon the combination of the Competitive Advantage on Assays (See section 4.4.6), Awareness of Company Assays (see above) and the Quality and Price Competitiveness (see Section 4.5).

4.4.5.2 Model Performance

The complicated structure of the dynamics of rivalry for the limited number of potential customers, based upon the Customer Win Potential (a factor of quality of service offered (reputation), knowledge of the existence of the company and services offered (marketing) and the competitive advantage held due to the assays available from in-house research and the public domain) is shown in Figure 4.7.
During the model development it became clear that one of the key assumption in the model is that there are a finite number of customers available, which can be both won form and lost to rivals. Customers can also be lost if they decide either that the assays available are very useful and thus expertise is worth developing in-house or that the assays are not useful and therefore they no longer require the service offered.

The finite availability of new customers increases the importance of repeat customers as shown by the steadily decreasing New Client %. Due to the significance of repeat customers, this has been discussed in depth in section 4.6.1.

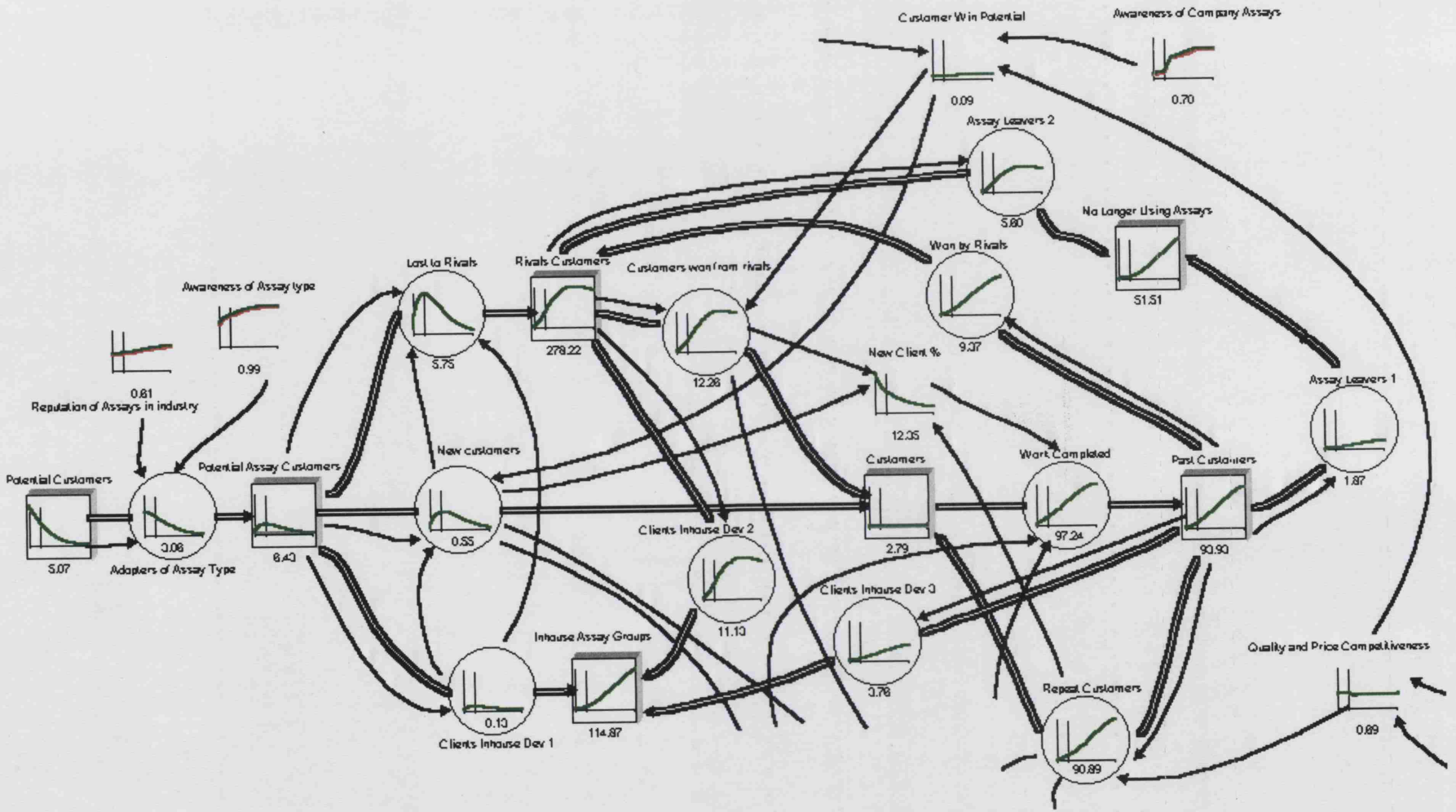


Figure 4.7: Customer base.

4.4.6 Mix of Assays

There are a number of assays in the public domain that could potentially be developed and offered to customers. Alternatively when the in-house experience grows, internal and novel assays could be offered to customers. Whilst some assays may be crucial to win customers, others may be more novel and require more aggressive marketing. Furthermore, the importance of offering a wide range of assays cannot be overstated. 34.1% of the two hundred people surveyed from the pharmaceutical industry (Roth, 2005) thought that a one-stop shop was very important (21.8% important) when choosing an outsourcing partner. Also the number of assays available to customers will affect the perceived quality of the service.

The mix of assays developed is shown in Figure 4.8.

4.4.6.1 Assumptions

Resources

- Potential Assays from public domain – It was assumed that there were 40 potential assays within the field of assays modelled that could be developed from the public domain in 2002.
- Partner's External Assays – The department modelled had a European research partner which was also developing assays within the field modelled. It was estimated that approximately half of all assays available from the public domain would fit better with the scientific expertise of this partner and thus would be developed overseas.
- Assays Developed Ext – This is the number of Assays that are developed and made available to customers as a service, from those which are available in the public domain.
- Potential In-house Assays – During development of Potential Assays available from the public domain and to meet the demand of customers and other areas of the company modelled, further potential Assays that may be developed will

be identified. Upon department set-up in 2002 and again in 2003, two such assay variants were developed.

- Assays Developed Int – These are the Assays developed using Internal knowledge and expertise.
- Years Assay Experience – As an Assay is used, experience of relevant handling and analytical techniques will develop. This will increase the Quality of work done as discussed in section 4.4.7 below.

Flows

- Published papers with Potential Assays – It was estimated that on average 2 further potentially reproducible assays are published each year, which would be developed an offered as part of the service.
- Assays Developed Int – Competitive Advantage is likely to be developed by the Unique Selling Point of having developed in-house assays. Furthermore many of the assays are expected to be developed to meet specific customer demand, with the importance of securing repeat customers already having been discussed. Therefore it has been assumed that in-house potential assays will be developed in preference to those developed from papers published in the public domain. In reality the decision on which Assay to develop next will be complex and critical to business success. As such these decisions are better made by a panel of informed management.
- Assays Developed Ext – As noted above, it has been assumed that time and analytical resources will be concentrated on Assays Developed Int. in preference to those based on information from the public domain. The remaining staff and analytical time available will then be spent developing Assays based on information published in the public domain.
- Company Developed Technical Knowledge – It was assumed that the idea for one potential assay that could be successfully developed as a customer Assay in each year, with none identified in 2004, and two identified in 2006 and 2008.

- Assays Developed by Partners – It has been assumed that 10% of assays available for development in the public domain will be developed by the European partner and therefore from a strategic perspective will not be developed by the department modelled.
- Rise in Experience – Experience on each assay available to customers is gained annually.
- Reduction in Experience – Any staff leaving would reduce the assay experience, however like staff leaving and analytical equipment redundancy this has been assumed to be nil in the model.

Variables

- R&D Time – see section 4.4.3
- Assays available to customers – This is the total number of assays available to customers, developed from either the public domain or based upon internal knowledge. Whilst Assays Developed Int have been prioritised as discussed above, the decision on which assays to develop and their worth (Net Present Value post development) is a very complex and specific assessment, therefore it has been assumed that each assay that is available is equally likely to increase the number of assays that any customer purchases and generates the same amount of revenue each time it is performed.
- Average Assay Experience/Av Experience Required/Assay Experience – These parameters have been introduced to model the effect that lacking experience on an assay can have on the quality of the assays produced. It has been estimated that it takes an average 3 months (0.25 of a year) of experience on an assay before it reaches an acceptable quality. In reality newly developed services are more likely to be offered to the first customer(s) at a reduced price, after the relative inexperience in its use has been explained.
- Competitive Advantage on Assays – Due to the importance of developing close links with customers, as demonstrated by the importance of repeat customers, many Contract Research Organisations attempt to set themselves up as a "One-stop shop" for their customers. It has been therefore been estimated that the level of competitive advantage gained is due to:

- 5% from the proportion of externally available assays that have been developed
- 25% from the proportion of internal assays developed
- 70% from the Effect of other company services.

- Researcher Time on Assay Dev – This indicates the researcher time spent on developing assays, based upon 20 days to develop an assay from the public domain and 40 days to develop an assay from internal knowledge.
- Cost – See section 4.5.

Factor

- Effect on Other Company Services - It has been assumed that the effect of other company services are a constant, with 90% of the potential advantage that they can offer being provided due to the relatively comprehensive services available elsewhere within the company.

Model Performance

- From the assumptions made, with maximum economic growth the department will be able to provide 28 assays it has developed from the public domain and 11 assays it has developed internally by 2010.

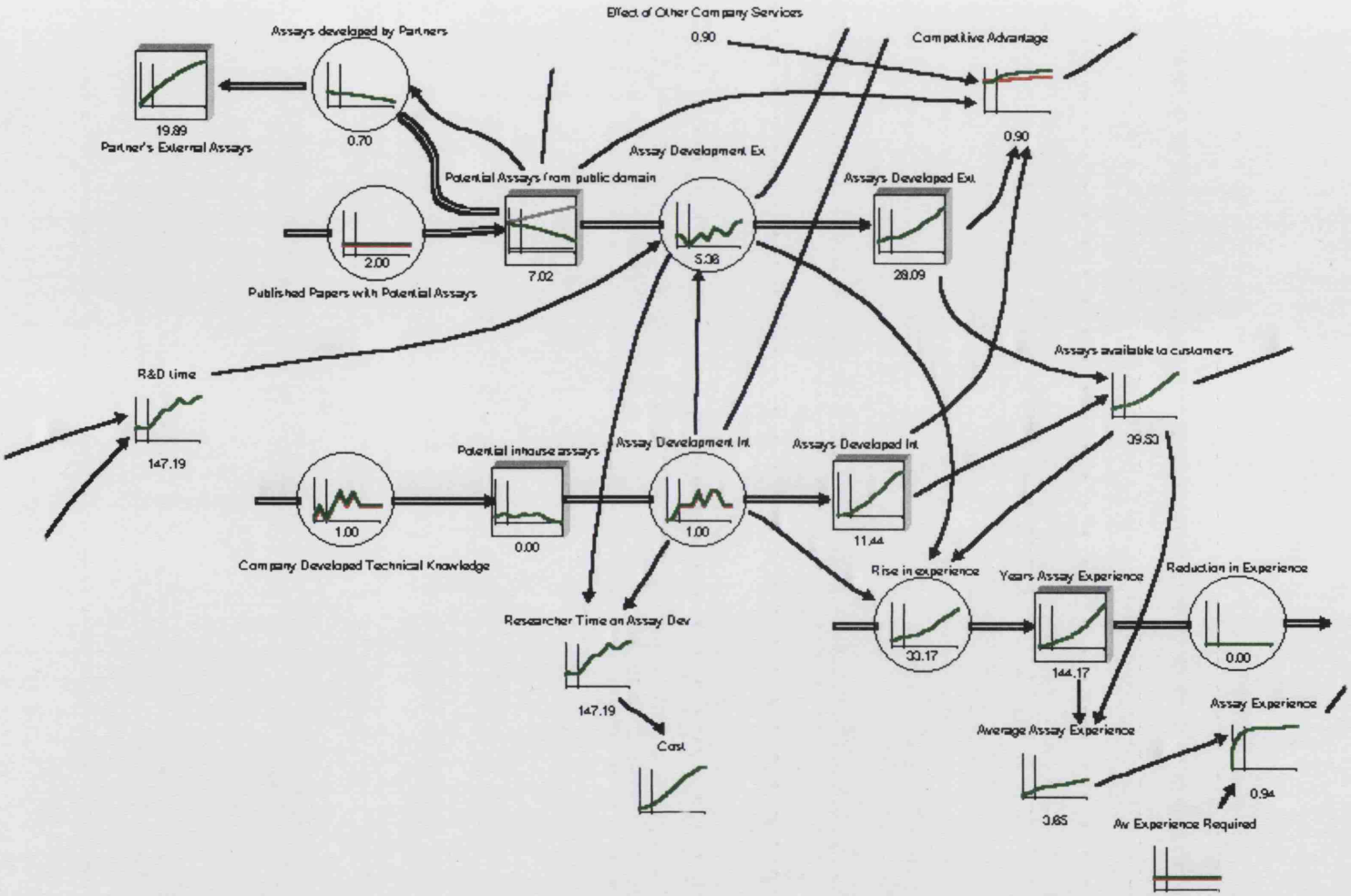


Figure 4.8: Assay development.

4.4.7 Quality of service

The key to a successful CRO is to provide a quality service that satisfies the needs of customers. If a company is willing to outsource experimentation to a CRO (be that due to lack of own resource, specialist knowledge and equipment or risk management), then they will only provide repeat custom if they are satisfied with the results. As already mentioned, many CROs are currently trying to operate a one-stop shop for pharmaceutical manufacture, and as such having the broadest array of assays available offers a stronger market position. However, incorporated into the quality is also the effect of having experience in that type of assay, the amount of researcher experience and the pressure on staff (low morale reducing the quality of the work performed).

The quality of the service is pivotal to the number of customers won and the amount of repeat business obtained, and thus the long-term effectiveness of the business and every effort should be made to provide the highest quality service possible. The drivers and effects of quality are shown in Figure 4.9.

4.4.7.1 Assumptions

Resources

- Potential Assays from public domain/ Assays Developed Ext./ Assays Developed Int. – See Section 4.4.5.1, Figure 4.8.

Flows

- Customers won from rivals / New customers / Repeat customers – See Section 4.4.5.1, Figure 4.7.

Variables

- Effect of Other Company Services – See section 4.4.6 above
- Competitive Advantage – See section 4.4.6 above
- Researcher Experience – See section 4.4.3 above.

- Assay Experience/Pressure on Staff/Quality of work done – see section 4.4.6 above.
- Reputation with Clients – Assumed to equal the Quality of work done.
- Cost Mark Up **<Management Handle>**/Industry average price/Relative Price – The cost model used in the department modelled is to re-charge customers based upon a mark-up of the costs incurred in performing the assays. This cost method has the advantage of being simple to calculate and easy for customers to understand, however it includes no benchmarking against the pricing of rivals. It is believed that the prices charges to customers is lower than that of competitors based on anecdotal feedback obtained, therefore it has been modelled that the industry average price charged is 14% more expensive on a relative basis. The high importance of the relative price and thus competitiveness with rivals warrants further investigation into the pricing strategy that is used across the rest of the industry and would allow benchmarking of the price charged.
- Quality and Competitiveness – It has been assumed the quality of service and price competitiveness are equally likely to influence repeat custom, with each factor having contributing 50% toward the overall attractiveness of the service.
- Awareness of Company Assays – see section 4.4.6 above.
- Customer Win Potential – see section 4.4.5.1 above.

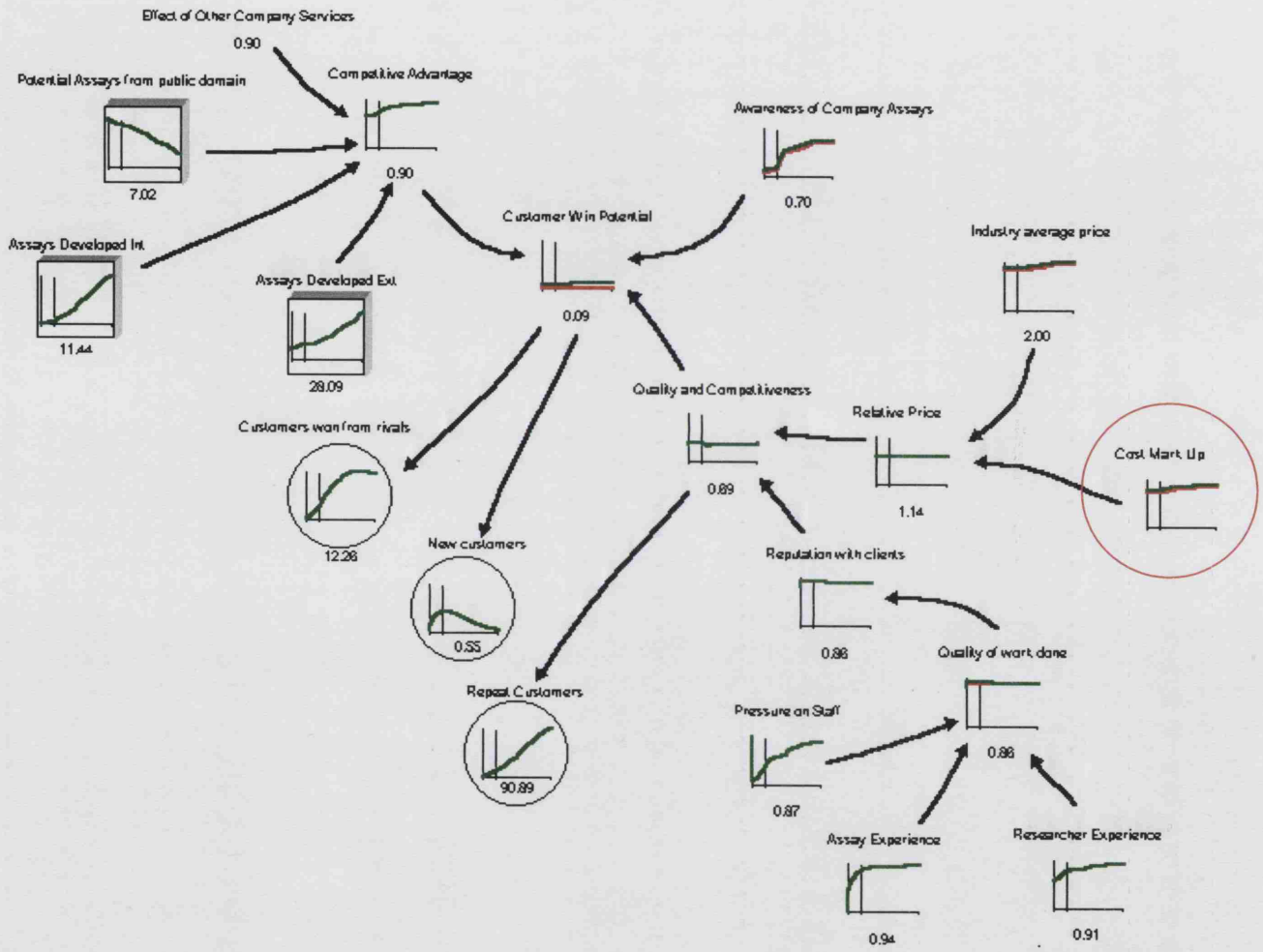


Figure 4.9: Quality of service provided where the red ring represents a key management handle.

4.5 Development cost and service revenue

With the high cashflow requirements associated with all research activity, the generation and utilisation of the available capital is crucial to overall success. Whilst cash is not a strategic resource, it was incorporated into the model to enable analysis of the effects of different strategic decisions on both short and long-term profitability.

Cash is generated by performing assays for customers and is used to increase the repertoire of assays available to customers and as working capital to perform further assays. The results of which are shown in Figure 4.10.

In the model, as based upon the historical pricing strategy, profit is achieved by performing assays and using a cost plus structure, i.e. assays were priced based upon a fixed percentage increase above the cost of performing each assay type. It is recognised that this may not promote the most competitive price:

- Assays which are simple to perform, but requiring expensive reagents may be uncompetitive.
- Those requiring a great deal of research effort to make available to customers, yet using cheap reagents may be over competitive, never repay the initial expenditure and customers could infer a lack of quality.

However, this pricing strategy has the advantage of being transparent to customers and as such is often found to be the preferred cost structure to customers. Note that the disadvantages above may be overcome by employing a development cost specific mark-up, but for this generic model such complexity is not warranted. Due to commercial sensitivities, the mark-up used (a key management handle) has been removed, although a steady increase is shown to reflect the increased demand resulting from an improved reputation over time. For similar reasons the cost revenue and gross margins have been removed.

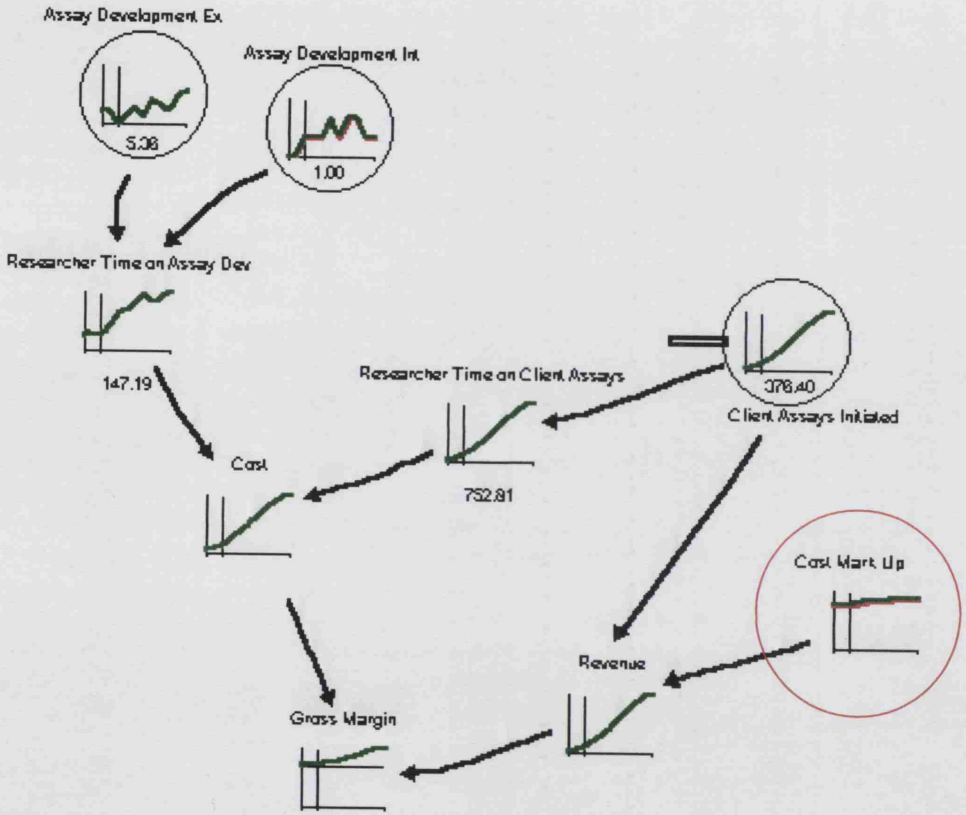


Figure 4.10: Cost and revenue where key management handles are represented by a red ring.

4.6 Model potential

The key characteristic (repeat custom) and the different uses of the model are discussed below.

4.6.1 Importance of repeat custom

Due to the limited number of potential customers, importance of repeat custom is of key concern. Figure 4.11 shows the percentage of work that is likely to be won from repeat custom. Whilst after one year of operation in 2004 around 50% of work was derived from repeat custom, this figure is expected to reach 80% by 2007 and should level off at around 90%. If customer satisfaction is low due to poor service quality then the result on the business will be potentially catastrophic (see section 4.6.3). Therefore every effort should be made to provide the best service possible.

Figure 4.12b gives an indication of the source of customers in 2012. As the total number of potential customers is relatively small and the estimation of this key parameter may be inaccurate, further investigation by market research could be crucial to the accuracy of the model and subsequently the strategic decisions and ultimate success of the CRO. The estimate here is based upon an expert in the field and in-house market research conducted with the company senior sales partners.

4.6.2 Growth strategy

The potential knowledge and decision making assistance derived from the generation of a model of the company resources has already been discussed. To assess the effect of different approaches to growth, two different investment scenarios have been studied:

One in which there is no further growth (Figure 4.12a). This could potentially occur due to a risk-averse management who are unwilling to seek external further investment; and

One in which maximum potential growth is sought, with no aversion to risk (figure 4.12b). Whilst this may appear optimistic, the potential benefit of making subsequent business decisions *in silico*, rather than making intuitive decisions in reality has already been described and is likely to lead to seemingly more risky strategies being taken.

Due to the often described importance of repeat customers, the maximum growth strategy involved winning as many new customers as possible early on and simultaneously aggressively researching new assays to offer, ensuring that resources key tangible resources of staff and analytical equipment never become limiting. The results of the two different scenarios are shown in Figure 4.12a and 4.12b, from which it can be seen that in either case there will be only either one or no new customers who have never used the assay type before. The number of customers won from rivals is also very similar (around thirteen) again showing how crucial repeat custom is to long term profitability. The number of repeat customers is over four-fold different from the different growth strategies taken and the number of assays in progress and thus revenue generated is over five-fold higher (the difference resulting from the increased number of assays requested by repeat customers compared with newly won customers).

The potential lack of new adopters of the assay type within a decade may also assist investment decisions to be made about the amount of caution exercised, business decisions about the benefit of long-term strategic partnerships, and the potential desirability of marketing to win new customers (not studied in the model).

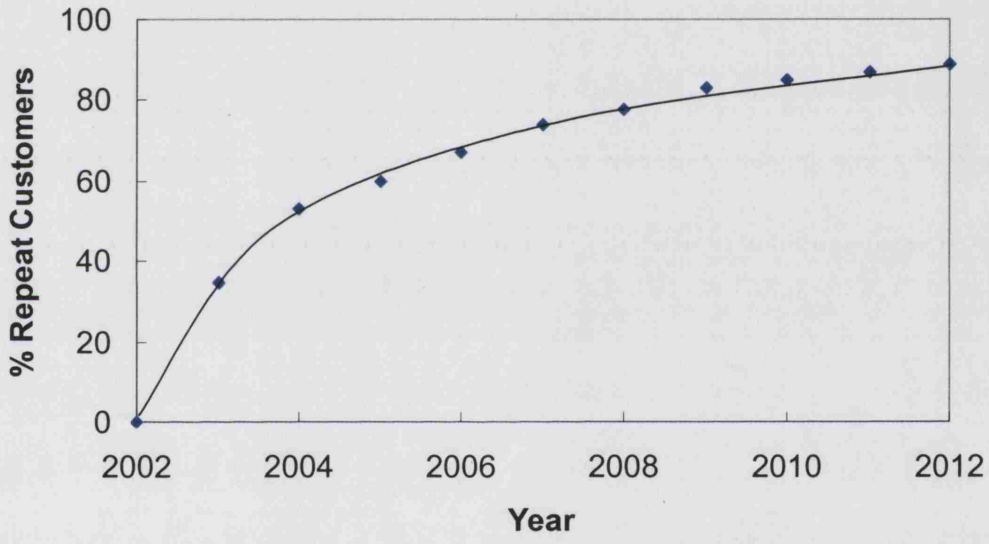


Figure 4.11: Percentage of work from repeat customers

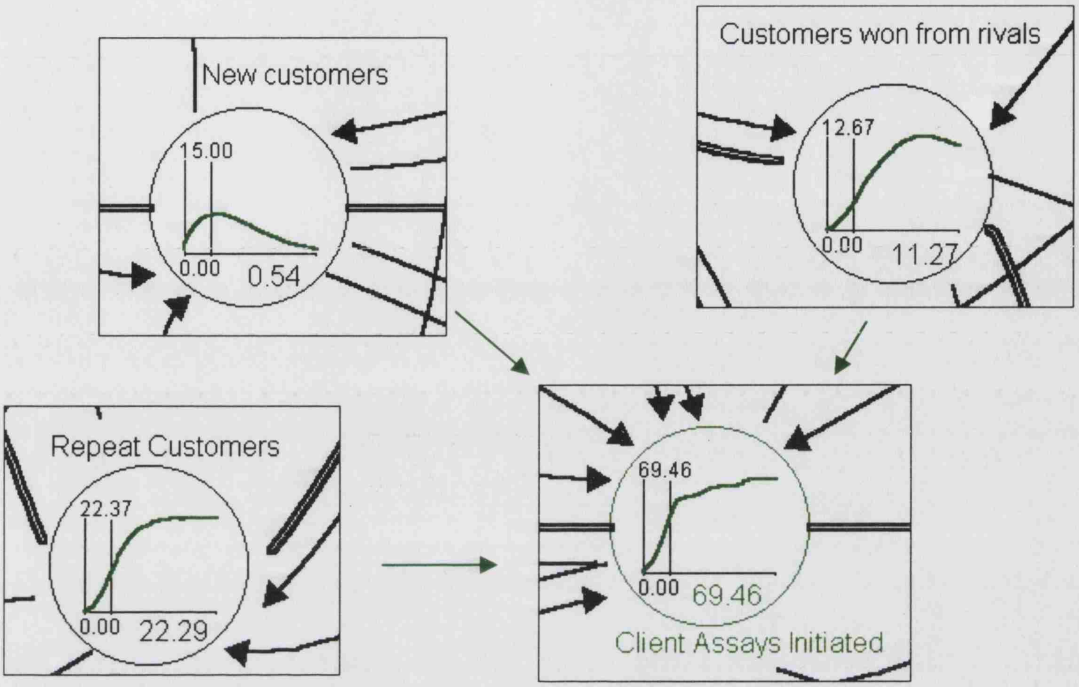


Figure 4.12a: Modelling Results: No further growth

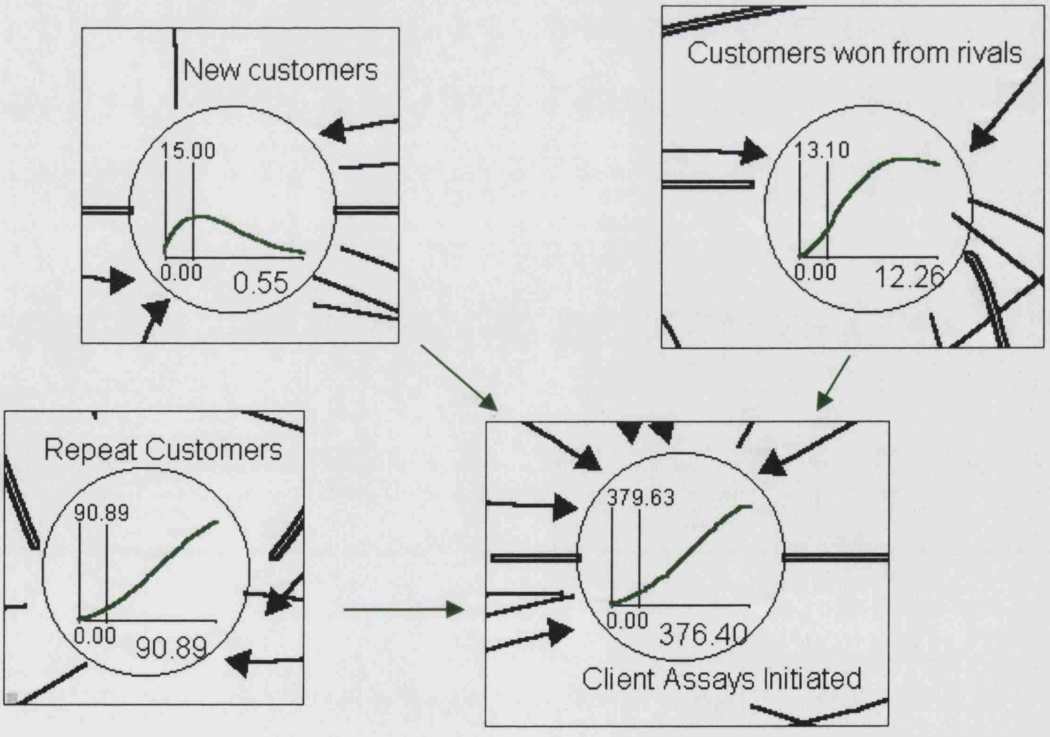


Figure 4.12b: Modelling Results: Maximum possible growth.

4.6.3 Scenario planning potential

Due to the importance of repeat customers and the impact that quality will have on this, to assess the potential utility of the model for scenario planning, the long term effect of a short term drop in quality was analysed. Such a short term drop in quality is not unrealistic and could feasibly happen for a number of reasons such as:

- A key member of staff may leave, taking with them the expertise required to perform certain assays and use certain equipment. To safeguard against this two people should be familiar with each type of assay.
- The analytical equipment may fail and it may not be possible to analyse assay results. To plan for such an outcome the potential to outsource the analytical part of the assay or to hire alternative equipment should be investigated.
- A computer virus or fire may destroy standard reporting templates. To prevent such an outcome regular system back-ups should be taken.

The results from a 75, 50 and 25% drop in quality (from its current average) are shown in Figure 4.13. The importance of repeat custom has already been explained, with 80% of work being won from repeat customers by 2007. Therefore the 10% drop in repeat custom that remains two years after a 75% drop in quality will represent an 8% drop in revenue and thus the amount available to spend on assay development and new equipment. Even after seven years the number of repeat customers will be over 2% lower than it would otherwise have been. Table 4.2 gives the effect of the quality drop on the number of repeat customers. Whilst these data may not be surprising, they would enable the best short-term strategic decisions to be made. For instance if over the next five years it is known that £250,000 of revenue will be lost, it is simple to decide that it is worth spending £50,000 hiring some new analytical equipment for six months. However without such a model the long-term effects of short-term decisions could not be tested and very tough, intuitive decisions would be required.

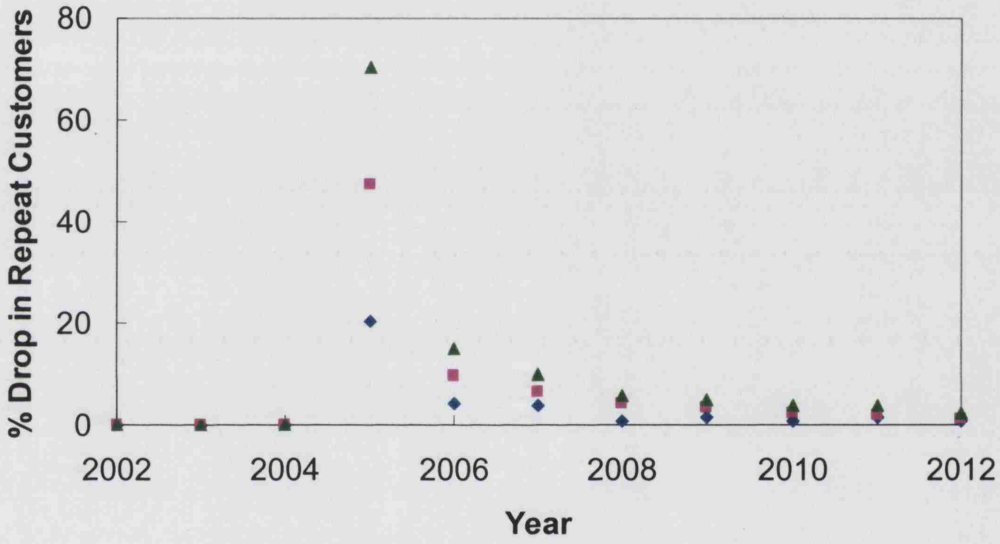


Figure 4.13: Scenario planning: Effect of a "quality event" on the percentage of repeat customers.

A drop in service quality, for the first six months of 2005, to 75% (▲), 50% (■) and 25% (◆) of its estimated current average, and the cumulative effect of this over time is shown.

Drop in Quality	Drop in repeat custom that year	Cumulative drop in repeat custom over following 7 years
25%	20%	34%
50%	47%	76%
75%	70%	116%

Table 4.2: The dynamic effect of a drop in quality on subsequent year repeat custom.

4.7 Conclusions

To both survive and prosper a start-up CRO needs to be able to balance everything. The analytical and research time spent on the development of the assay range needs to be balanced against the time spent performing contract work. The mix of well known assays that are demanded by customers and the potentially improved and competitive advantage of generating in-house assays that are developed must be carefully balanced. Too few standard assays will give a perception of lack of expertise and may discourage new customers, similarly too few in-house assays may indicate to potential clients a lack of ability to innovate.

The risk of different growth options needs to be balanced against the potential reward of each strategy. The cash generated needs to be balanced against strategic growth needs. The experience of the staff recruited balanced against the cost and benefit of such experience.

The quality of the assay is critical to success and the speed at which the number of assays available to customers increases is crucial to gaining market share in a relatively small overall customer base. A trade off between rapid development and quality development must be made. Due to the importance of maintaining an image of quality (to ensure repeat custom from a relatively small potential pool), it is suggested that this is the over-riding factor, and the potential loss of some market share can be absorbed.

Where CROs need to be at the forefront of the technology to enable them to keep gaining new clients, and when client in-house assay potential becomes more widely spread for older assays then the situation becomes complex indeed. Simple strategic decisions can seem almost impossible to base anything on other than intuition and business experience. However Strategy Dynamics modelling represents an alternative approach where a continually growing and evolving model is created that can not only be used to make long term strategic decisions, but can also be used to simulate different potential business decisions.

The benefits of dynamic modelling are:

- It allows more knowledgeable management decisions to be made
- Key strategic decisions can be tested *in silico* prior to being put into practice in the real world.
- Decisions can be made more confidently at earlier times to increase potential profits.

Whilst the results may not always be a perfect mirror of the outside world and the accuracy will depend upon the complexity and effort of the model, the evolutionary process will hone and improve the mathematical inter-relationships and parameter estimation used. Section 5.3 describes how such evolution improvements can be made by model validation.

One factor that has been mentioned, but not discussed due to its commercial sensitivity is that of price competition. Setting too high a price can result in lack of customers whereas too low a price can indicate a lack of quality and lead to loss of funds crucial to the development of further assays. Fluctuating prices associated with testing the market can also lead to customer disillusionment. Such pricing decisions can be difficult to make without knowledge of the likely customer numbers and without a discernable strategy. The modelling detailed here can assist by allowing both an appropriate and reasonable return on investment to be calculated based on different possible scenarios. This should aid in setting a reasonable service price and help to lessen the risk of insolvency.

5 Aspects of Validation

5.1 Introduction

The regulatory pressures on the pharmaceutical industry are increasing. This is predominantly for two reasons (Borradaile, 1997):

1. As a method for governments to control their healthcare costs.
2. To increase the standard of healthcare and the efficacy of pharmaceuticals.

Unknown properties of approved drugs are continuing to damage the reputation and profitability of the drugs industry. The recent voluntary withdrawal of antidepressant Vioxx by Merck, after it was shown to double the chances of heart attacks and strokes, is expected to lead to $10-15bn of lawsuits, which has raised the awareness of drug safety to unprecedented levels (Frantz, 2005). Subsequent to this costly withdrawal and other rumours of incomplete clinical trial disclosure, the US have proposed the 'Fair Access to Clinical Trials Act' of 2005 to require pharmaceutical companies to disclose to the public all new clinical drug trials data (olpa.od.nih.gov). Whilst increasing freedom of such information may provide patients with improved choice, a lack of scientific knowledge within the majority of the population means that they are unlikely to be able to interpret the results of these studies without significant levels of guidance.

Validation is derived from the need to understand what is expected from a process. The FDA prescribes that a standard validation protocol is used. This is in the form of a written plan stating how the validation will be carried out and includes the following key stages:

- Identification of test parameters and product characteristics
- Description and conditions used for processing equipment used
- Evaluation of critical process parameters and their operating ranges
- The number of validation runs, including the sampling and test data to be collected
- An explanation of decision points on what constitutes acceptable test results

According to Frank Matarrese, director of compliance at Chiron Corp. (Emeryville, CA, USA), the ideal number of validation runs to determine the worst case scenario for general process parameters is three (Glaser, 2001). With one run at the high, middle and low-end of the control range or three high and three low to show that the process works at the edges of the control range.

Whilst the development of traditional drugs is strictly regulated, those from biologically derived sources are subject to even stricter compliance rules.

Validation of the reagents used is required for Current Good Manufacturing Practice (CGMP) – where the inter-batch variation seen with biologically derived complex molecules, that are sensitive to changes in operational conditions, makes them critical reagents (Ritter and Wiebe, 2001).

Validation is primarily the identification of the sources of potential errors and the subsequent quantification of them. It is important as it describes the performance characteristics of a process mathematically, in quantifiable terms. It is important to note that a validated process is not necessarily tightly controlled or well run.

The report published by CDER (FDA) in 1998 entitled "Guidance for industry: Bioanalytical Methods Validation for Human Studies" stated that validated assays are now required for all endpoint parameters in a clinical trial.

Validation, as a measure of accuracy has relevancy to each chapter studied so far. Generally where the result of processes is human consumption, there is a high degree of regulation. Where a process is later in the development process, and thus has a higher impact on the overall result, the regulatory burden is higher and more closely monitored.

5.2 Regulatory bodies and sources of information

Information on validation is available from the two predominant regulatory bodies, the European Medicines Evaluation Agency (EMEA) and the US Food and Drug

Administration (FDA). The Center for Biologics, Evaluation and Research (CBER) and the Center for Drug Evaluation and Research (CDER) are branches of the FDA that are of particular relevance to the biotechnology industry. Though rules and guidance is given by the Medicines Control Agency (MCA) and information is provided on the sterile manufacture of drugs by the Parental Drug Association (PDA).

5.3 CHMO

As discussed within Chapter 1, due to the chirality introduced by biochemical synthesis routes the CHMO bioconversion of cyclic ketones can yield potentially attractive pharmaceutical intermediates. As the products are intermediates are not biologically derived drugs themselves, they would not require detailed validation and product purity is higher importance. However as the EngD requires a chapter on validation to be included within the thesis, the principles have been considered below.

Biological assays can lack reproducibility and can show low precision. Table 5.1 shows the industry precision standards for both enzyme-based and cell-based assays. The inclusion of the cell wall and complex metabolism substantially reduces the precision of the assay. By potentially including an immobilisation step, the precision is likely to be further reduced.

Assay type	Average precision
Enzyme based assay	<10%
Cell based assay	25%

Table 5.1: Industry precision standards for relevant assays as given quoted in CBER guidelines (http://www.fda.gov/cber/summaries/120600bio10.htm).

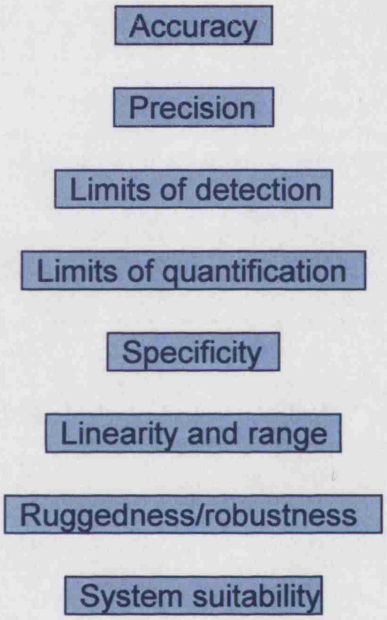


Figure 5.1: The eight validation assay parameters, as defined by CBER guidelines (http://www.fda.gov/cber/summaries/120600bio10.htm)

There are three validation tests for the quantitative determination of assays:

1. Repeatability – the reliance that can be place on the results, based on the range for the procedures, e.g. the range of substrate concentrations.
2. Intermediate Precision – the effect of random events on the precision, e.g. different days, analysts, equipment, etc.
3. Reproducibility – important for the standardisation of an analytical procedure, usually studied as an inter-laboratory test.

As whole cell oxygenase biocatalysts are infrequently immobilised, the repeatability of the immobilisation process has been assessed by the considering the reaction rates yielded. Figure 5.2 shows the results of ten repetitions of a standard experimental assay using immobilised whole cells. The co-efficient of variance of ±12% compares favourably with the average precision values quoted for cell based assays and as such the repeatability of immobilised whole cell reactions is not considered problematic.

5.4 Scale-up of metabolite production

The production of drug metabolites as potential new drug candidates and for studies of drug-drug interactions, is likely to find use in late stage drug development, and as such is likely to be subject to regulatory compliance guidelines.

Changes in processes, equipment or materials need to be managed in a systematic approach to maintain validation control. Due to the reliance on liver microsomes in the assessment of the CYP metabolising enzyme, a relevant aspect of validation for a potential metabolite production scale-up service would be validation change control, i.e. the robustness of the assay to different suppliers of microsomes and sources of NADPH regenerating solution.

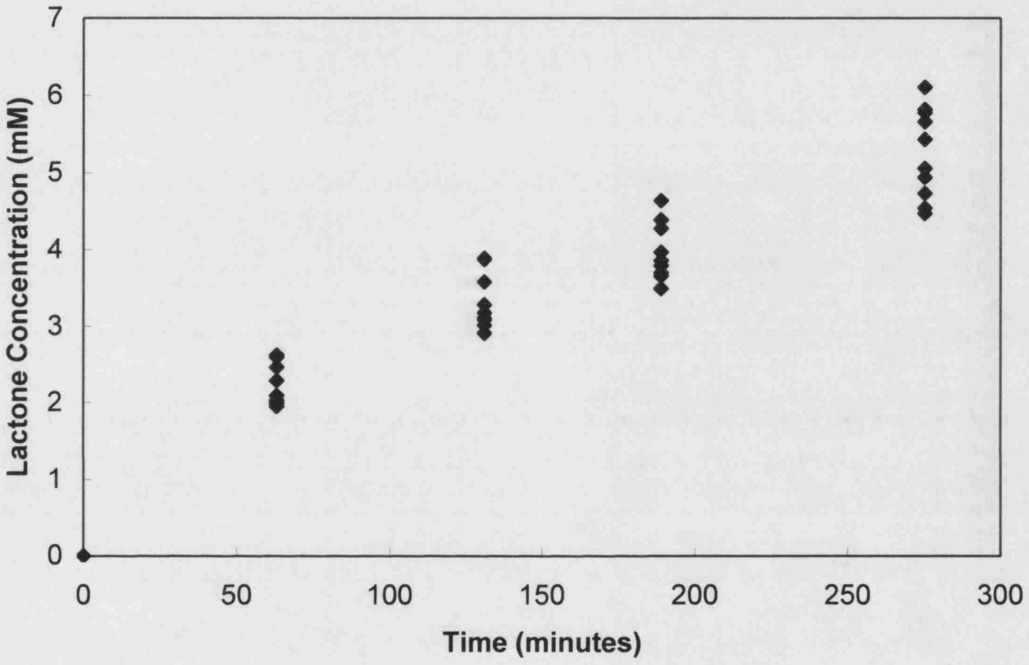


Figure 5.2: Repeatability of ten immobilised whole cell reactions with bicyclo[3,2,0]hept-6-en-2-one. The results show a coefficient of variance of ±12%.

In vitro Technologies gives a recommended method of preparing NRS and states that it may be used either fresh or frozen; this method was given in section 3.2.3.1. BD Biosciences recommends that their own NRS solution be used, with two components being mixed prior to use.

The results of the identification of the metabolising CYP enzyme are from experiments using BD Biosciences liver microsomes and the IVT method of preparing NRS. Figures 5.3 shows the smooth reaction profiles resulting from the metabolism of verapamil and diltiazem by BD Biosciences rat liver microsomes with the different methods of preparing NRS. All three types (*In vitro* Technologies fresh and frozen, and BD Biosciences) are suitable for metabolic studies and yield broadly similar rates of metabolism. The short lag shown with frozen NRS in the verapamil metabolism suggested caution should be used with frozen NRS, however generally it appeared suitable. In fact the metabolism of diltiazem with this frozen solution was marginally faster than that seen with the fresh solution. The pre-weighed BD Biosciences NRS was relatively slower at regenerating the cofactor for metabolism and thus NADPH is more likely to be rate limiting in this system. From a validation perspective, the feasibility of the different NRS is likely to maintain assay robustness.

The change to IVT liver microsomes by comparison is likely to have greater effect. Figure 5.4 shows comparable results to those for BD Biosciences in Figure 5.3, but for IVT rat liver microsomes. Whilst the results for verapamil appear only slightly less reliable, for diltiazem large inter-sample variations are seen. The extent of the variability is likely to prevent reliable analysis of the rate of metabolism and if the assay were validated, complete reassessment of the validation is likely to be required were a change to IVT liver microsomes necessitated.

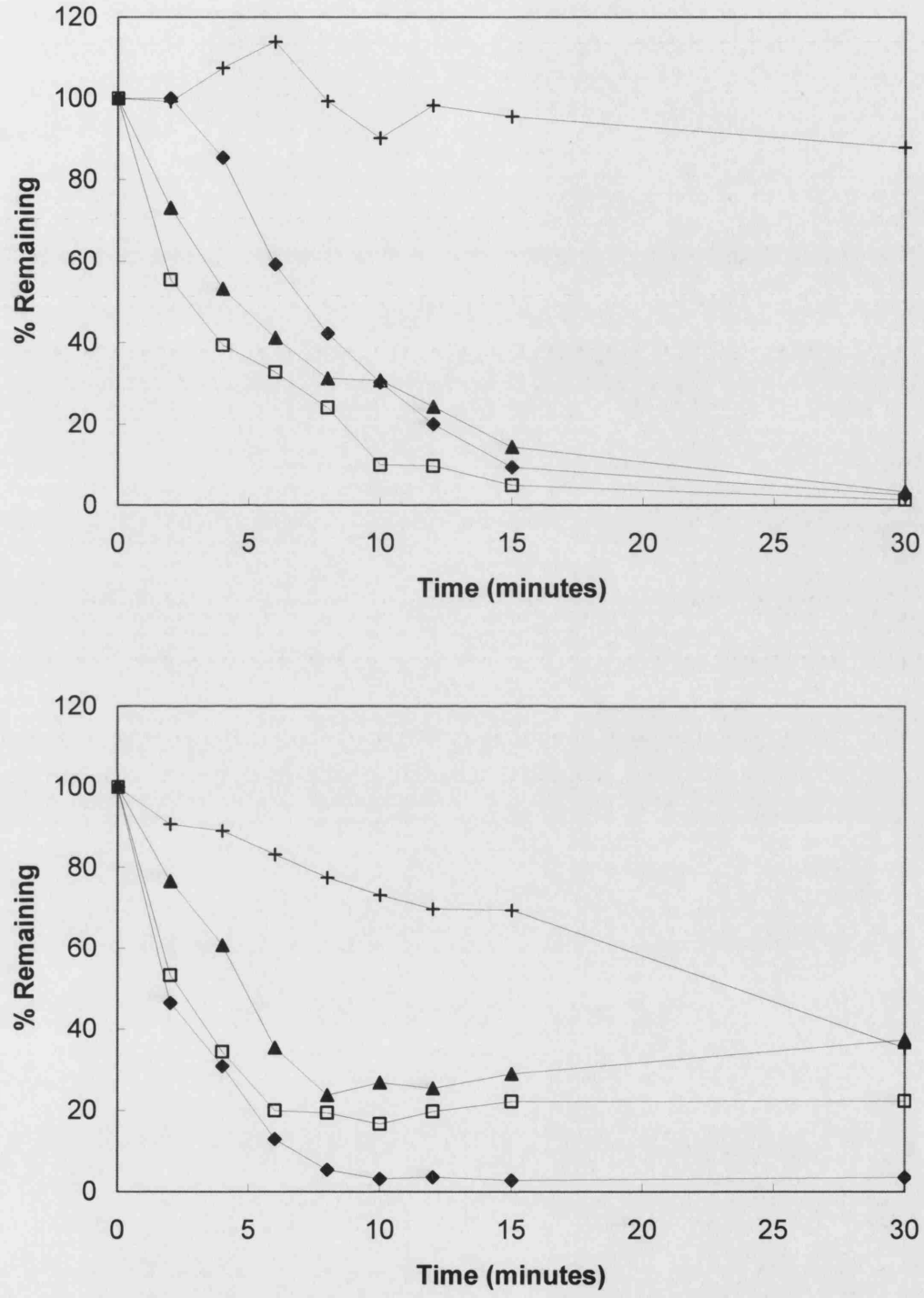


Figure 5.3: Comparison of the source of NADPH regenerating system using the metabolism of verapamil (Top) and diltiazem (Bottom) with BD Biosciences rat liver microsomes.

◆ Frozen *In vitro* Technologies NADPH regenerating system
□ Fresh *In vitro* Technologies NADPH regenerating system
▲ Fresh BD Biosciences NADPH regenerating system
+ No NADPH regenerating system

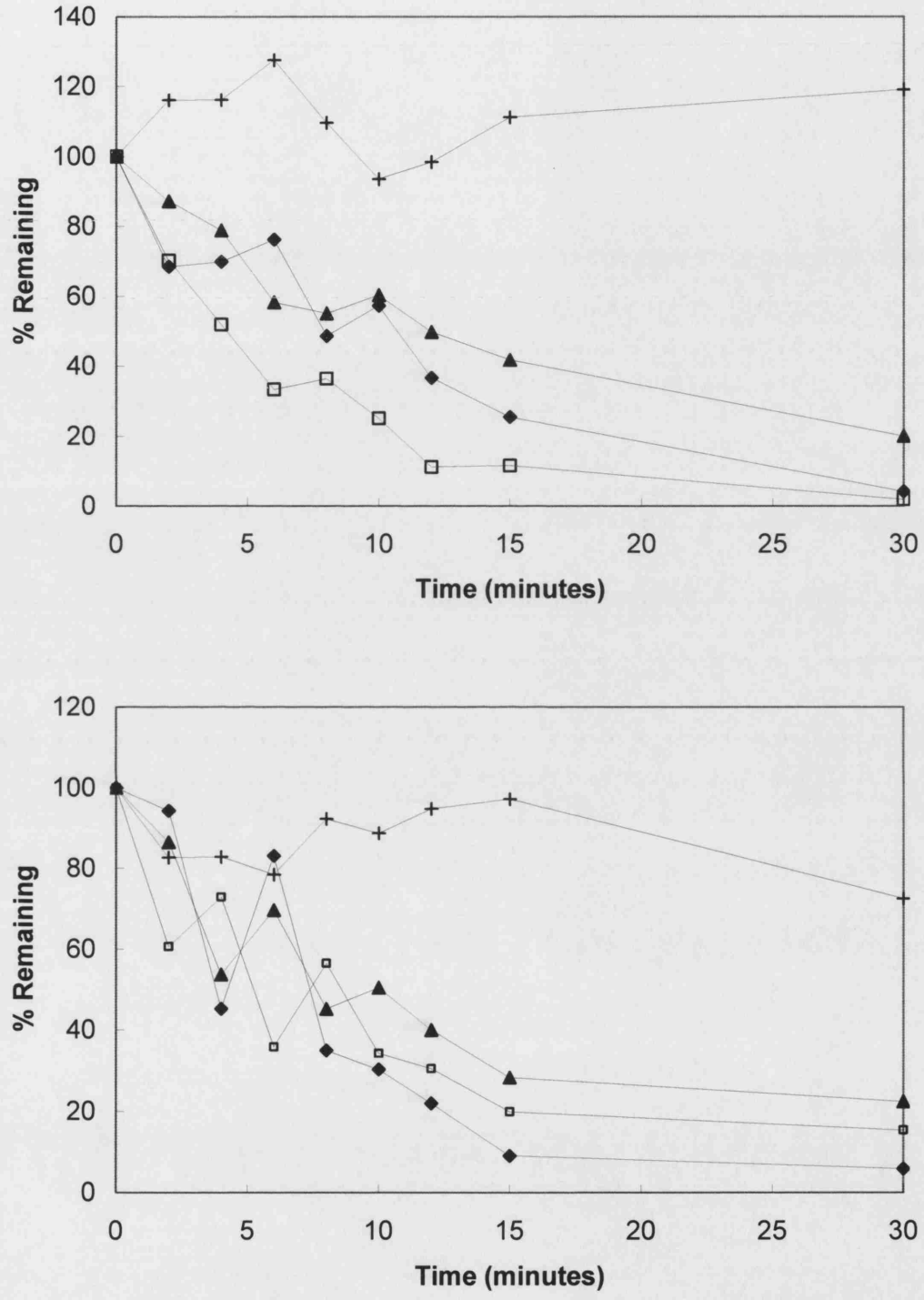


Figure 5.4: Comparison of the source of NADPH regenerating system using the metabolism of verapamil (Top) and diltiazem (Bottom) with *In vitro* Technologies rat liver microsomes.

◆ Frozen *In vitro* Technologies NADPH regenerating system
□ Fresh *In vitro* Technologies NADPH regenerating system
▲ Fresh BD Biosciences NADPH regenerating system
+ No NADPH regenerating system

5.5 *Strategy dynamics of a start-up CRO*

The strategy dynamics model of a start-up contract research organisation is subject to less formal validation requirements. However as described in chapter four the relevancy of the model is closely linked to its accuracy.

In 2002 the FDA (The department of health and human services, FDA, 2002) found that of 3140 medical device recalls conducted between 1992 and 1998, 7.7% were attributable to software failures. 79% of which were caused by software defects that were introduced when changes were made to the software after its initial production and distribution. Whilst such software applications are significantly different to the model derived here, it indicates the importance of validating model adjustments subsequent to the described performance monitoring. The predominant benefit of software validation according the FDA report on software validation is increased usability and reliability.

Due to the predictive nature of the model it will only be accurate if the inter-relationships studied are robust. Whilst two years of data from a new department of an CRO have been used to build the model and test it, to ensure that it has good predictive ability, the key strategic resources should be graphically recorded over time and any management decisions made should be also made within the model (i.e. hiring of staff). Table 5.2 gives potential methods by which the different strategic resources can be measured.

Strategic resource	Dependent upon	Measured by
Repeat customers	Assay quality	Benchmarking industry standards (time taken, quality of reporting, accuracy, price, etc.) Customer surveys sent with research results (perceived quality, likelihood of repeat business). Timeliness of results delivery. Customer complaints
	Other company services available (i.e. level of control held)	Consumer trends (e.g. Contract Pharma outsourcing reports)
	Total number of potential customers	Market growth (e.g. new companies registered at Company House)
Assays developed	Mix of assays developed from internal and external sources.	Customer surveys sent with research results (other services desired, usefulness of results provided, etc.)
Staff time	Experience	Number of assays that each staff member can perform and the number of times each assay has been performed. Individual utilisation rates
Analytical equipment time	Availability	Equipment down time, sample throughput, time of uninterrupted operation.

Table 5.2: Potential methods of recording strategic resource flows.

In addition to the validation of the dynamic strategic model, plotting graphs of the key strategic resources over the coming years and subsequent update of the model would allow:

- Improved business awareness, market knowledge and subsequent increased levels of intuition in decisions made without the use of the model.
- Improved ability to de-bottleneck operations in advance, i.e. hiring of research staff and purchase of analytical equipment.
- Improved project management and research efficiency by defining limiting steps.
- More accurate information into the quality of service and its effect on repeat custom.
- Assessment of the developing market allowing more specific marketing and pricing models to be implemented.
- The potential demand for in-house assays compared to assays developed from the public domain.

5.6 Summary

The validation of pharmaceuticals processes is critical to the safe manufacture of drugs and thus to ensure their efficacy and to safeguard human life. There are several aspects of validation that are required prior to the successful completion of the clinical trials of drugs. The relative importance of each parameter is dependent upon type of process and the stage of the process in the development and manufacture of drugs. The precision of the immobilisation process for CHMO biocatalysis and the potential lack of availability of suitable sources of liver microsomes for the metabolite identification process were highlighted as key parameters and have been considered here.

Validation of the strategy dynamics model has similarities with that of pharmaceutical processes. To be an effective tool for aiding management with strategic decision making the results must be reliable and useable. An effective validated model should help to preserve and enhance the profitability of the business.

6 Future work

6.1 CHMO biocatalysis

1. Work comparing the effects of inhibition on the two alternative CHMO host (E. coli TOP10 [pQR239] and JM107) was performed prior to the introduction of flow cytometry at UCL. Due to the relatively similar inhibition profiles seen it would be interesting to re-perform this work with the inclusion of flow cytometric analysis. This would enable proof as to whether it was the cell physiology or the over-expression of CHMO which was responsible for the slight differences seen in inhibition observed.

2. To test the longevity of the immobilised whole cells of *E. coli* TOP10, the ISSSPR experiments carried out by Simpson *et al.* (2001) should be repeated to allow assessment of whether the stability of the enzyme is significantly increased by immobilisation of the whole cell host, i.e. whether the enzyme is stable for more than sixteen hours.

3. The study of different host cells suggested that inhibition was not simply reliant on substrate and product concentration levels, but is also dependent upon the length of time at which the cells are in contact with a substrate or product at a given concentration. This effect was supported, though to a lesser extent, by the inhibition profiles seen with the isolated enzyme bioconversions where lower inhibition levels were observed with these two minute reactions compared with the sixty minutes free cell reactions where the cytoplasmic membrane of the cell protects the enzyme. To further clarify this effect, the increasing different substrate inhibition studies should be repeated at the 2L scale to determine whether prolonged exposure to a set substrate concentration gives similar trends as those seen in the shaken flasks. To enable this very close control of the ketone feed pump levels will be required which may require the use of Near Infra-Red (NIR) spectrographic monitoring (Bird *et al.*, 2002).

4. It has been demonstrated that immobilised whole cell reaction rates can be increased by reducing the beads size. Further assessment of the potential for reaction rate improvements can be made by utilising an industrially machined bead making device. The high specification construction would promote bead size homogeneity at higher gas flow rates enabling the reliable production of beads at diameters of circa 10μm. This should enable further increased reaction rates which are similar to those seen in free cells, however the effect of reduced availability of oxygen for cell metabolism and respiration need to be assessed as this appeared to affect the concentration at which substrates/products became inhibitory for faster reacting substrates. The inhibitory profiles observed with the larger bead sizes should be repeated with small beads to allow further quantification of the effect of time on the level of inhibition.

5. A potential method of further increasing the reaction rate beyond the levels observed in these studies would be to permeabilise the cell membranes (for example with solvents as was described by Canovas *et al.*, 2005). Immobilisation may then appear more beneficial, with the support matrix providing the enzyme stability currently provided by the cell wall but with potentially faster diffusion of substrates, products and oxygen. By initially using flow cytometry to study the effect on cell physiology of different concentration of solvents, a narrower range of concentrations may be selectable prior to any study into the effect on reaction rate.

6. The lack of correlation between the inhibitory level of the substrate and respective product for the two commercially available lactones is a potentially interesting result given the similar structures. Further study could be carried out on the other lactones by using the chemical synthesis routes to these lactones as was used for the synthesis of GC analytical standards.

6.2 *Scale-up of drug metabolite production*

1. No inhibition of the metabolism of verapamil was apparent with furafylline, the selective inhibitor of CYP1A2. Whilst this is known to be less important than CYP3A4, the result shows how relatively unimportant CYP1A2 is. To assess the amount of metabolism shown by CYP1A2, bactosomes™ expressing only this enzyme should be tested to assess the effectiveness of this technique in finding all potential metabolising enzymes.

2. As no cell lines expressing either individual CYPs or over-expressing generic CYPs were commercially available, it was not possible to test the scale-up of metabolite production. To enable such testing, a cell line should be constructed or an agreement formed with one of the groups that have reported CYP expression in recombinant cells as described in section 1.3.4.3.

6.3 *Strategy Dynamic of a Start-up CRO*

1. The validation of the model by mapping key variables is the best method of improving the accuracy and thus predictive capability of the model.

2. To develop the model to an increasing level of complexity some of the simplifying assumptions (i.e. that all assays available in the public domain are equally important to potential clients) could be reassessed based on market research and more assay specific relationships introduced.

3. Due to the limited number of potential customers and the importance of repeat custom that was consequently found during the modelling process, market research could be conducted to find any other potential factors which may affect this key variable.

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Appendix I: Standard fermentation profiles

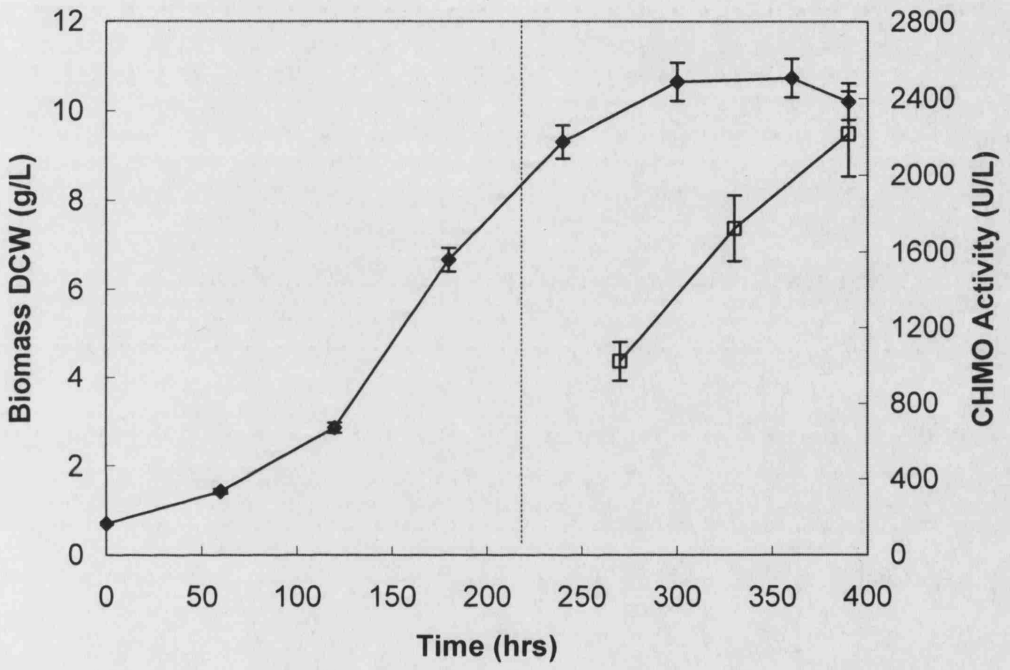


Figure AI.1: The growth kinetics and CHMO induction profile of a typical aerobic batch fermentation of *E .coli* TOP10 [pQR239]. Where:

◆ Biomass concentration and □ specific CHMO activity.

The dotted line indicates the point of CHMO induction, the fermentation was harvested for subsequent bioconversion experiments at 385 minutes.

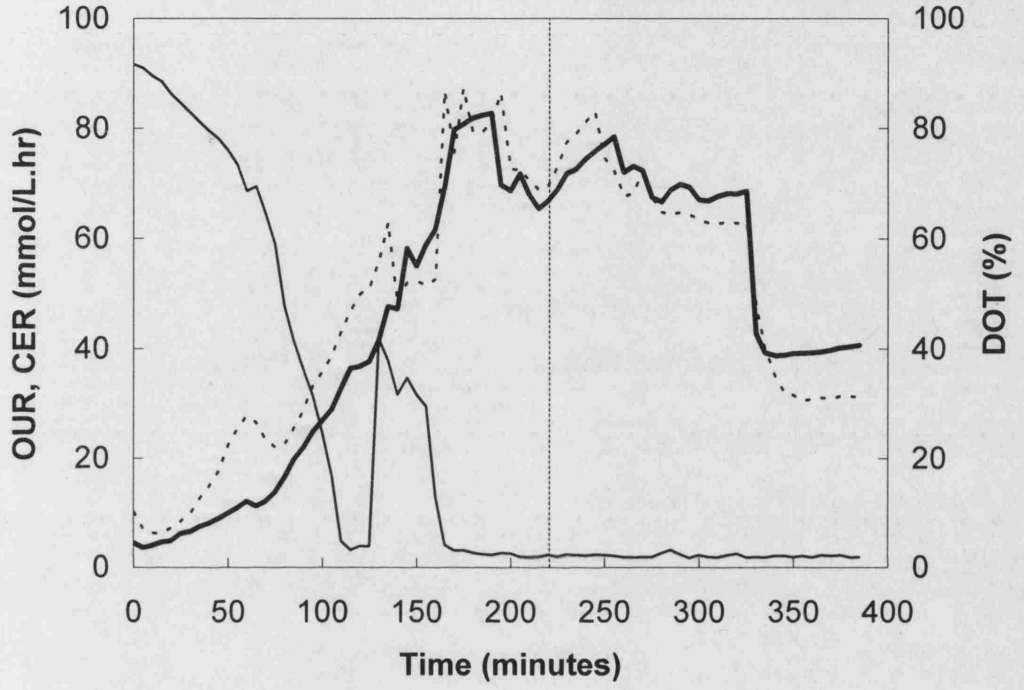


Figure AI.2: Exit gas data showing the corresponding OUR (—), CER (—), and DOT (- - -) profiles for the aerobic batch fermentation of *E. coli* TOP10 [pQR239] shown in Figure A1.1. The fermentation and induction were performed as described in Section 2.3. Exit gas data was logged as described in Section 2.2.2.3. The increase in DOT in the fermenter after 120 minutes was the result of an increase in the impeller speed, as described in Section 2.2.2.3. The dotted line indicates the point of CHMO induction and the fermentation was harvested for subsequent bioconversion experiments at 385 minutes.

Appendix II: DSP figures

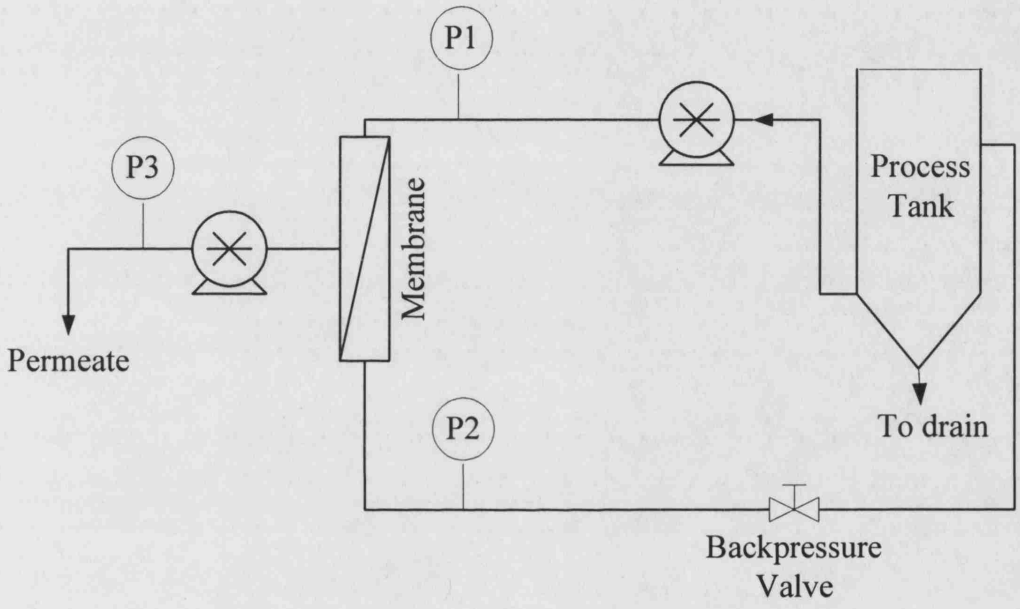


Figure AII.1: Schematic of crossflow filtration rig.

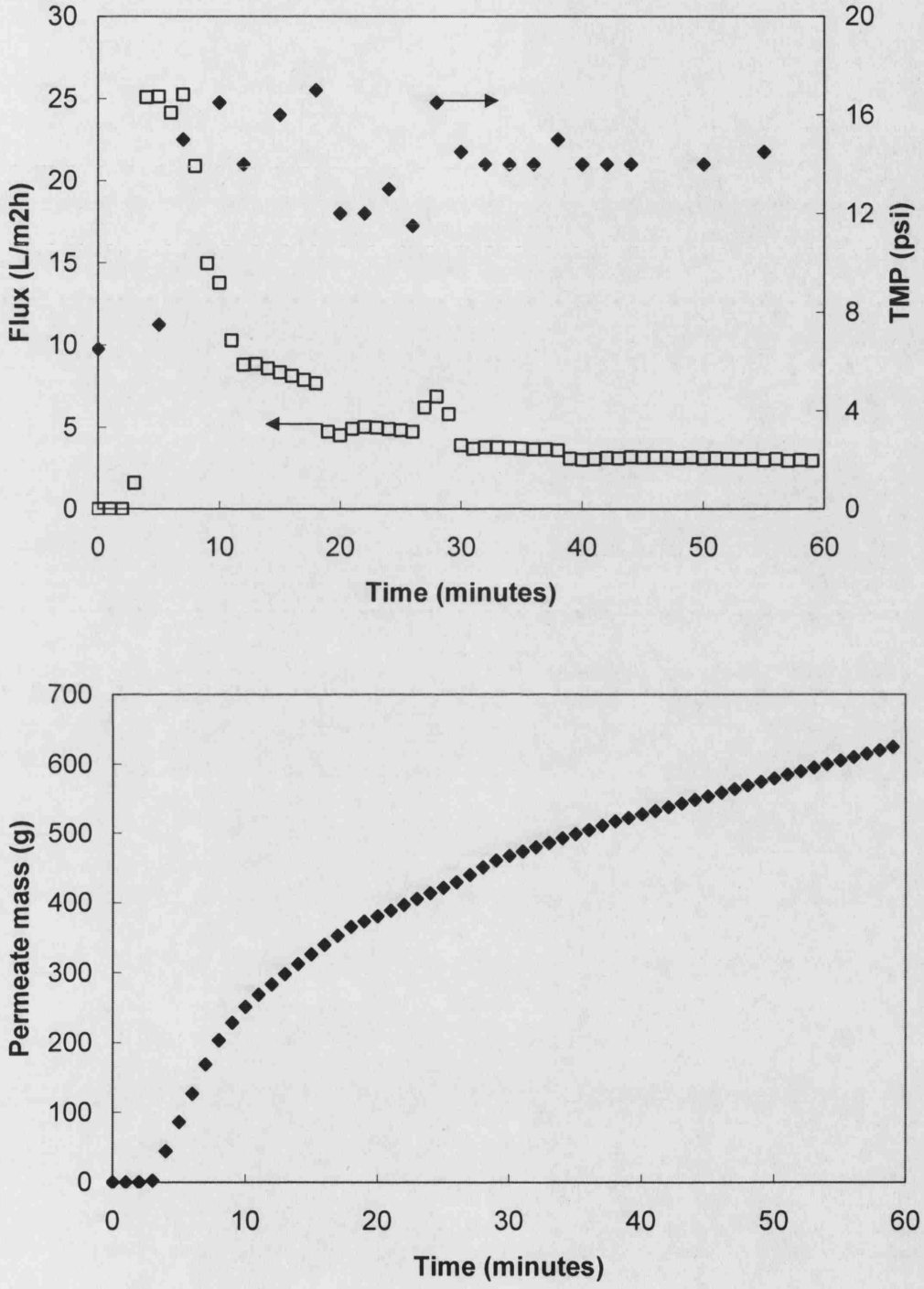


Figure AII.2: Microfiltration performance: Change in trans-membrane flux and pressure over time (Top) and permeate mass over time (Bottom).

Appendix III: Calculation of the intracellular CHMO activity by spectrophotometer.

The assay by spectrophotometer that was used to measure the activity of isolated CHMO is described in Section 2.2.3. A sample curve from the spectrophotometer is shown in Figure III.1.

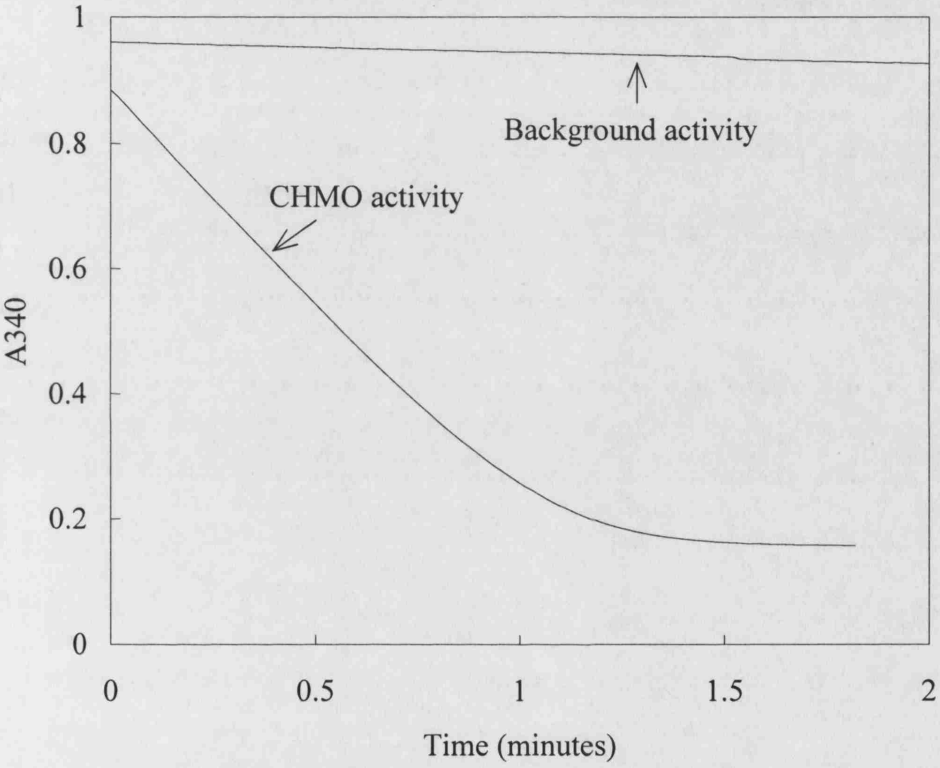


Figure III.1: The background absorbance and the adsorbance after addition of the cyclohexanone substrate for a typical spectrophotometric assay of isolated CHMO activity at 340nm. Assay performed as described in Section 2.2.3.

The activity of the enzyme extract was expressed in Units (U) with one unit being defined as the μmoles of NADPH consumed per minute. The calculation of the concentration of NADPH in the reaction cuvette was based on Beer's Law (Equation III.1).

$$A_{340} = \varepsilon\, c\, l \qquad \text{(III.1)}$$

A_{340} is the adsorbance at 340 nm, ε is the extinction coefficient of NADPH (6.22 mL/(μmol.cm)), c is the concentration of NADPH in the cuvette (μmol/mL) and l is the lightpath wavelength (cm). The activity of the sample and the background activity can therefore be determined using:

$$\text{Number of Units in the cuvette} = \frac{\Delta A_{340}}{l\varepsilon\Delta t} \qquad \text{(III.2)}$$

In the example shown in Figure III.1 the initial linear rate of absorbance change after addition of the substrate, is 0.691 ΔA_{340}/min. Subtraction of the background rate of 0.017 ΔA_{340}/min gives 0.674 ΔA_{340}/min. From Equation III.2 the number of units in the cuvette can be calculated to be 0.1084 U/mL. As a 1 mL cuvette is 1 ml and a dilution factor of 20 is routinely applied, the activity of the cell extract is calculated to be 2170 U/L. The biomass concentration in the fermentation medium is 10 g/L so the specific intracellular CHMO titre is 217 U/g.

Appendix IV: Oxygen limitation in shaken flasks

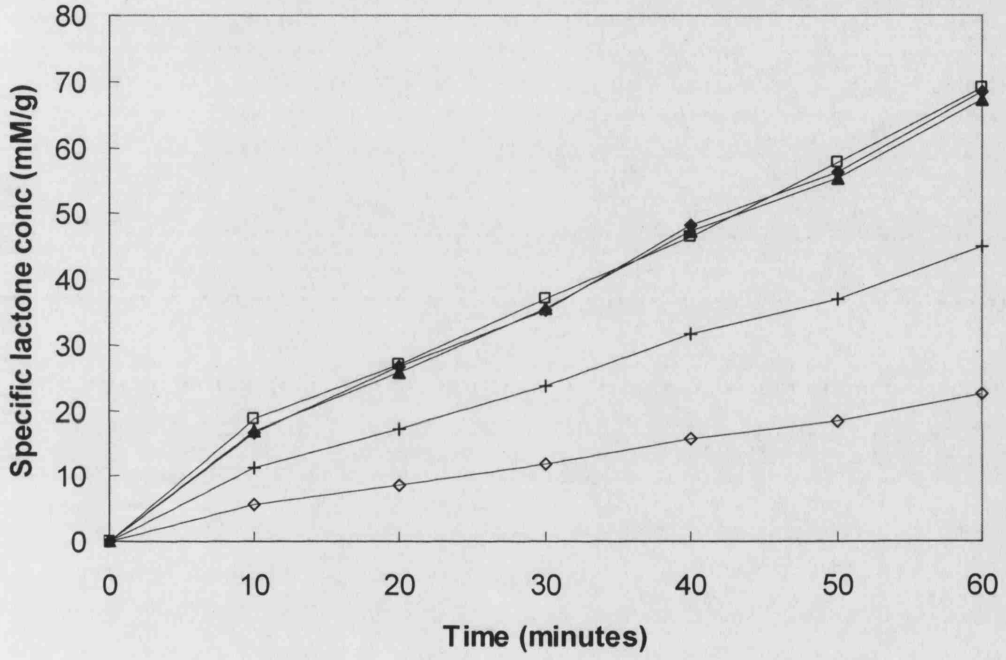
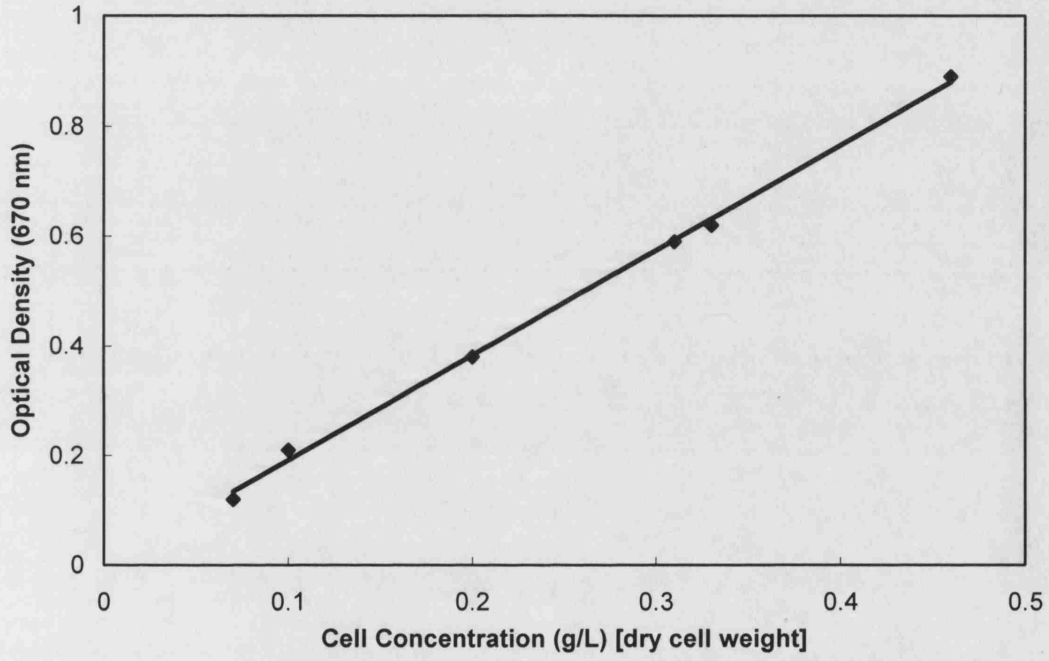


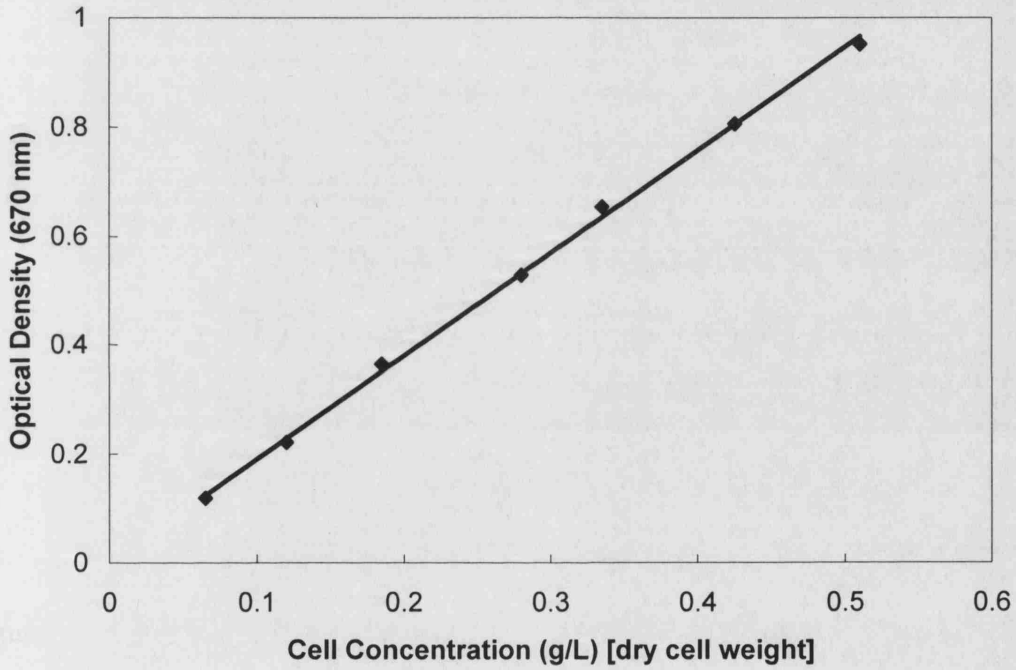
Figure IV.1 – Lactone produced in shaken flask experiments for varying cell concentrations (g (DCW)/L):

◆ 0.5, □ 1, ▲ 2, + 4, ◇ 8

Appendix V: Cell density calibration curves for *E. coli* TOP10 and *E. coli* JM107.



Appendix V.1: *E. coli* TOP10 [pQR239] spectrophotometer calibration curve. Where $y = 1.9137x$ and $R^2 = 99.8\%$.



Appendix V.2: *E. coli* JM107 spectrophotometer calibration curve. Where $y = 1.8951x$ and $R^2 = 99.9\%$.

Appendix VI: Volatility of 4-propyl cyclohexanone

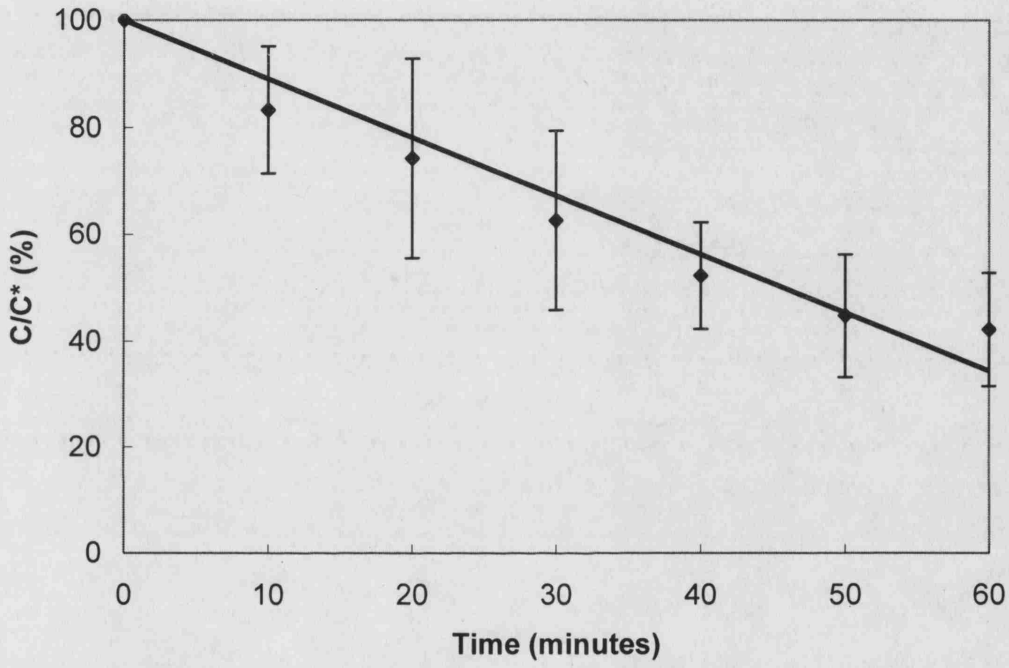


Figure AVI.1: The volatility of 4-propyl cyclohexanone. Standard 1 hour shake flask study with no biocatalyst at three substrate concentrations (20-50mM).
Where C/C* is the percentage of the concentration at any given time against the initial concentration. Error bars represent one standard deviation.

Appendix VII: Potential bead scale up equipment

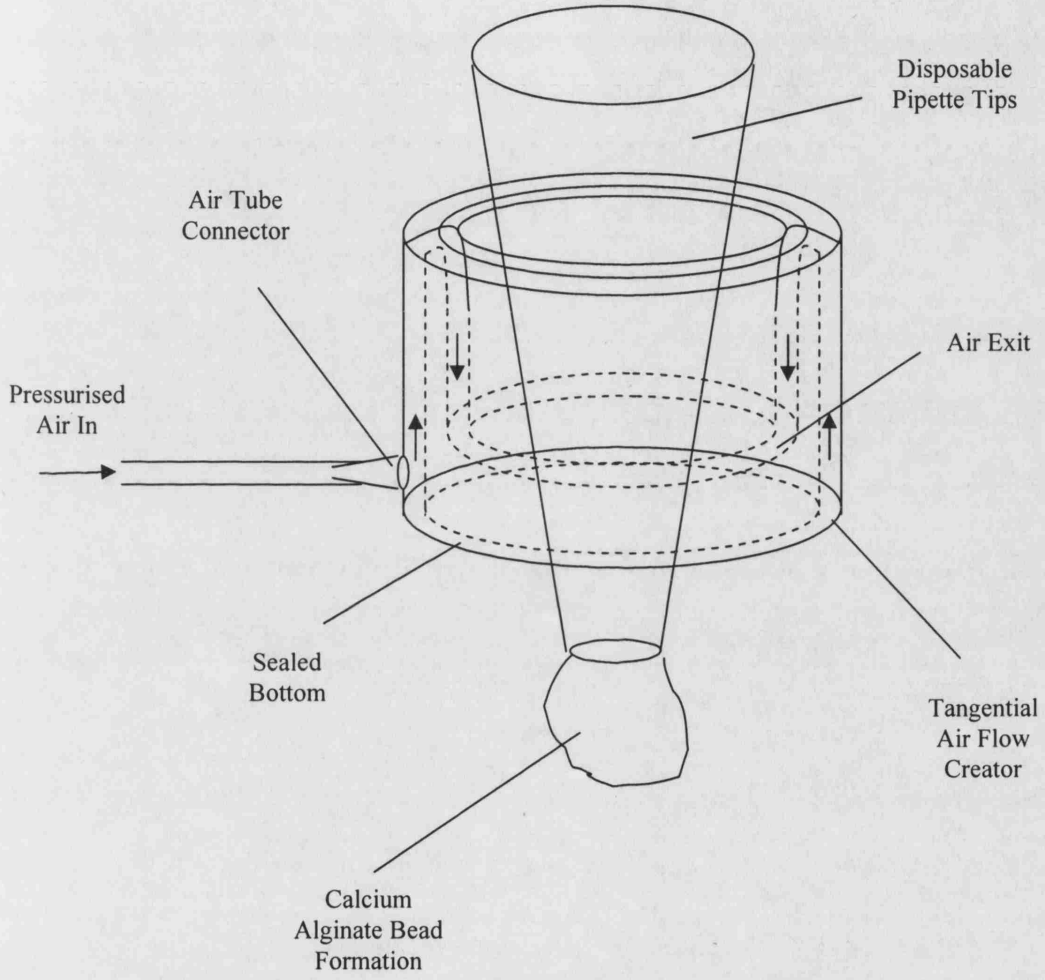


Figure AVII.1: Close up diagram of a potential scale-up calcium alginate immobilised *E. coli* bead making device.

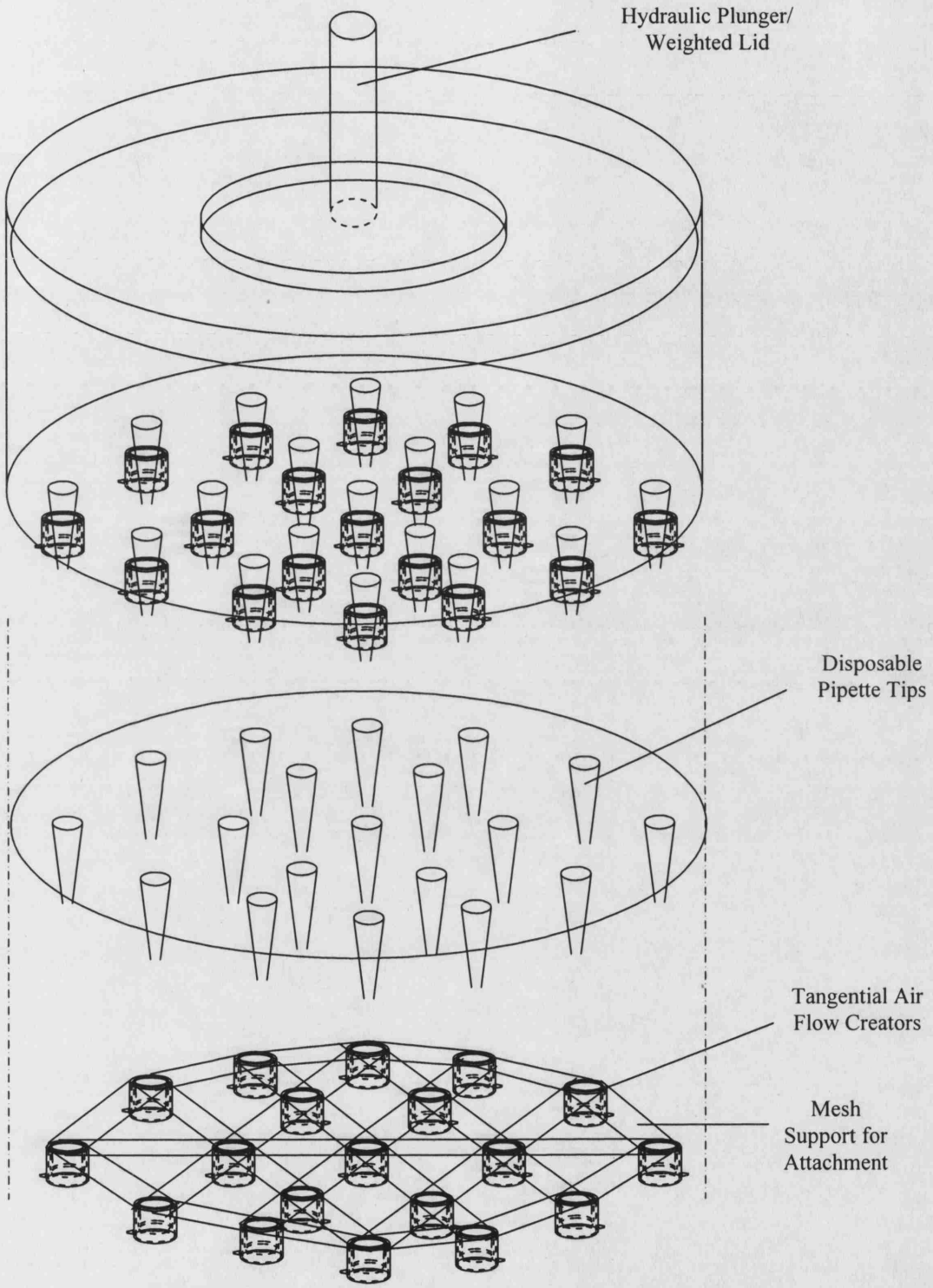


Figure AVII.2: Diagram of a potential scale-up bead making device.